(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 July 2004 (08.07.2004)

PCT

(10) International Publication Number
WO 2004/056998 A1

(51) International Patent Classification[7]: C12N 15/52, 9/00, 9/02, 9/10, C07K 14/21, C12Q 1/68, C12N 15/63, 15/53, 15/54, C12P 17/12, C07D 471/22, 471/18, A61K 31/4995, A61P 35/00, 31/04

(21) International Application Number:
PCT/GB2003/005563

(22) International Filing Date:
19 December 2003 (19.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0229793.5 20 December 2002 (20.12.2002) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/056998 A1

(54) Title: THE GENE CLUSTER INVOLVED IN SAFRACIN BIOSYNTHESIS AND ITS USES FOR GENETIC ENGINEERING

(57) Abstract: A gene cluster has open reading frames which encode polypeptides sufficient to direct the synthesis of a safracin molecule.

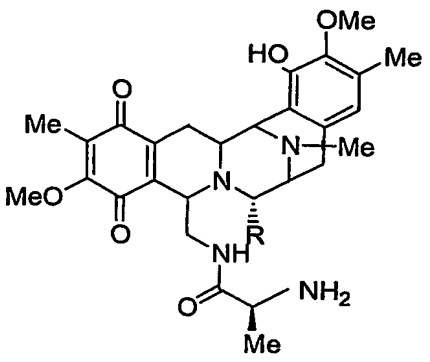
THE GENE CLUSTER INVOLVED IN SAFRACIN BIOSYNTHESIS AND ITS USES FOR GENETIC ENGINEERING

FIELD OF THE INVENTION

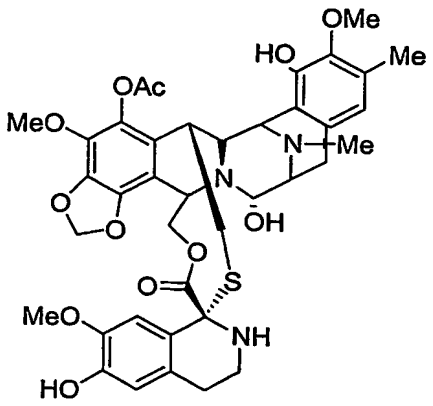
The present invention relates to the gene cluster responsible for the biosynthesis of safracin, its uses for genetic engineering and new safracins obtained by manipulation of the biosynthesis mechanism.

BACKGROUND OF THE INVENTION

Safracins, a family of new compounds with a potent broad-spectrum antibacterial activity, were discovered in a culture broth of *Pseudomonas* sp. Safracin occurs in two *Pseudomonas* sp. strains, *Pseudomonas fluorescens* A2-2 isolated from a soil sample collected in Tagawagun, Fukuoka, Japan (Ikeda et al. *J. Antibiotics* 1983, 36,1279-1283; WO 82 00146 and JP 58113192) and *Pseudomonas fluorescens* SC 12695 isolated from water samples taken from the Raritan-Delaware Canal, near New Jersey (Meyers et al. *J. Antibiot.* 1983, 36(2), 190-193). Safracins A and B, produced by *Pseudomonas fluorescens* A2-2, have been examined against different tumor cell lines and has been found to possess antitumor activity in addition to antibacterial activity.



Safracins A R=H
B R=OH



ET-743

Due to the structural similarities between safracin B and ET-743 safracin offers the possibility of hemi-synthesis of the highly promising potent new antitumor agent ET-743, isolated from the marine tunicate *Ecteinascidia turbinata* and which is currently in Phase II clinical trials in Europe and the United States. A hemisynthesis of ET-743 has been achieved starting from safracin B (Cuevas et al. *Organic Lett.* 2000, 10, 2545-2548; WO 00 69862 and WO 01 87895).

As an alternative of making safracins or its structural analogs by chemical synthesis, manipulating genes of governing secondary metabolism offer a promising alternative and allows for preparation of these compounds biosynthetically. Additionally, safracin structure offers exciting possibilities for combinatorial biosynthesis.

In view of the complex structure of the safracins and the limitations in their obtention from *Pseudomonas fluorescens* A2-2, it would be highly desirable to understand the genetic basis of their synthesis in order to create the means to influence them in a targeted manner. This could increase the amounts of safracins being produced, because natural

production strains generally yield only low concentrations of the secondary metabolites that are of interest. It could also allow the production of safracins in hosts that otherwise do not produce these compounds. Additionally, the genetic manipulation could be used for combinatorial creation of novel safracin analogs that could exhibit improved properties and that could be used in the hemi-synthesis of new ecteinascidins compounds.

However, the success of a biosynthetic approach depends critically on the availability of novel genetic systems and on genes encoding novel enzyme activities. Elucidation of the safracin gene cluster contributes to the general field of combinatorial biosynthesis by expanding the repertoire of genes uniquely associated with safracin biosynthesis, leading to the possibility of making novel precursors and safracins via combinatorial biosynthesis.

SUMMARY OF THE INVENTION

We have now been able to identify and clone the genes of safracin biosynthesis, providing the genetic basis for improving and manipulating in a targeted manner the productivity of *Pseudomonas* sp., and using genetic methods, for synthesising safracin analogues. Additionally, these genes encode enzymes that are involved in biosynthetic processes to produce structures, such as safracin precursors, that can form the basis of combinatorial chemistry to produce a wide variety of compounds. These compounds can be screened for a variety of bioactivities including anticancer activity.

Therefore in a first aspect the present invention provides a nucleic acid, suitably an isolated nucleic acid, which includes a DNA sequence (including mutations or variants thereof), that encodes non-ribosomal peptide synthetases which are responsible for the biosynthesis of safracins. This invention provides a gene cluster, suitably an isolated gene cluster, with open reading frames encoding polypeptides to direct the assembly of a safracin molecule.

One aspect of the present invention is a composition including at least one nucleic acid sequence, suitably an isolated nucleic acid molecule, that encodes at least one polypeptide that catalyses at least one step of the biosynthesis of safracins. Two or more such nucleic acid sequences can be present in the composition. DNA or corresponding RNA is also provided.

In particular the present invention is directed to a nucleic acid sequence, suitably an isolated nucleic acid sequence, from a safracin gene cluster comprising said nucleic acid sequence, a portion or portions of said nucleic acid sequence wherein said portion or portions encode a polypeptide or polypeptides or a biologically active fragment of a polypeptide or polypeptides, a single-stranded nucleic acid sequence derived from said nucleic acid sequence, or a single stranded nucleic acid sequence derived from a portion or portions of said nucleic acid sequence, or a double-stranded nucleic acid sequence derived from the single-stranded nucleic acid sequence (such as cDNA from mRNA). The nucleic acid sequence can be DNA or RNA.

More particularly, the present invention is directed to a nucleic acid sequence, suitably an isolated nucleic acid sequence, which includes or comprises at least SEQ ID 1, variants or portions thereof, or at least one of

the s*acA, sacB, sacC, sacC, sacD, sacE, sacF, sacG, sacH, sacH, sacI, sacJ, orf1, orf2, orf3* or *orf4* genes, including variants or portions. Portions can be at least 10, 15, 20, 25, 50, 100, 1000, 2500, 5000, 10000, 20000, 25000 or more nucleotides in length. Typically the portions are in the range 100 to 5000, or 100 to 2500 nucleotides in length, and are biologically functional.

Mutants or variants include polynucleotide molecules in which at least one nucleotide residue is altered, substituted, deleted or inserted. Multiple changes are possible, with a different nucleotide at 1, 2, 3, 4, 5, 10, 15, 25, 50, 100, 200, 500 or more positions. Degenerate variants are envisaged which encode the same polypeptide, as well as non-degenerate variants which encode a different polypeptide. The portion, mutant or variant nucleic acid sequence suitably encodes a polypeptide which retains a biological activity of the respective polypeptide encoded by any of the open reading frames of the safracin gene cluster. Allelic forms and polymorphisms are embraced.

The invention is also directed to an isolated nucleic acid sequence capable of hybridizing under stringent conditions with a nucleic acid sequence of this invention. Particularly preferred is hybridisation with a translatable length of a nucleic acid sequence of this invention.

The invention is also directed to a nucleic acid encoding a polypeptide which is at least 30%, preferably 50%, preferably 60%, more preferably 70%, in particular 80%, 90%, 95% or more identical in amino acid sequence to a polypeptide encoded by any of the safracin gene cluster open reading frames *sacA* to *sacJ* and *orf1* to *orf4* (SEQ ID 1 and genes encoded in SEQ ID 1) or encoded by a variant or portion thereof. The polypeptide suitably retains a biological activity of the respective polypeptide encoded by any of the safracin gene cluster open reading

frames.

In particular, the invention is directed to an isolated nucleic acid sequence encoding for any of SacA, SacB, SacC, SacD, SacE, SacF, SacG, SacH, SacI, SacJ, Orf1, Orf2, Orf3 or Orf4 proteins (SEQ ID 2-15), and variants, mutants or portions thereof.

In one aspect, an isolated nucleic acid sequence of this invention encodes a peptide synthetase, a L-Tyr derivative hidroxylase, a L-Tyr derivative methylase, a L-Tyr O-methylase, a methyl-transferase or a monooxygenase or a safracin resistance protein.

The invention also provides a hybridization probe which is a nucleic acid sequence as defined above or a portion thereof. Probes suitably comprise a sequence of at least 5, 10, 15, 20, 25, 30, 40, 50, 60, or more nucleotide residues. Sequences with a length on the range 25 to 60 are preferred. The invention is also directed to the use of a probe as defined for the detection of a safracin or ecteinascidin gene. In particular, the probe is used for the detection of genes in *Ecteinascidia turbinata.*

In a related aspect the invention is directed to a polypeptide encoded by a nucleic acid sequence as defined above. Full sequence, variant, mutant or fragment polypeptides are envisaged.

In a further aspect the invention is directed to a vector, preferably an expression vector, preferably a cosmid, comprising a nucleic acid sequence encoding a protein or biologically active fragment of a protein, wherein said nucleic acid is as defined above.

In another aspect the invention is directed to a host cell transformed with one or more of the nucleic acid sequences as defined above, or a

vector, an expression vector or cosmid as defined above. A preferred host cell is transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the assembly of a safracin or safracin analog. Preferably the host cell is a microorganism, more preferably a bacteria.

The invention is also directed to a recombinant bacterial host cell in which at least a portion of a nucleic acid sequence as defined above is disrupted to result in a recombinant host cell that produces altered levels of safracin compound or safracin analogue, relative to a corresponding nonrecombinant bacterial host cell.

The invention is also directed to a method of producing a safracin compound or safracin analogue comprising fermenting, under conditions and in a medium suitable for producing such a compound or analogue, an organism such as *Pseudomonas* sp, in which the copy number of the safracin genes/cluster encoding polypeptides sufficient to direct the assembly of a safracin or safracin analog has been increased.

The invention is also directed to a method of producing a safracin compound or analogue comprising fermenting, under conditions and in a medium suitable for producing such compound or analogue, an organism such as *Pseudomonas* sp in which expression of the genes encoding polypeptides sufficient to direct the assembly of a safracin or safracin analogue has been modulated by manipulation or replacement of one or more genes or sequence responsible for regulating such expression. Preferably expression of the genes is enhanced.

The invention is also directed to the use of a composition including at least one isolated nucleic acid sequence as defined above or a modification thereof for the combinatorial biosynthesis of non-ribosomal

peptides, diketopiperazine rings and safracins.

In particular the method involves contacting a compound that is a substrate for a polypeptide encoded by one or more of the safracin biosynthesis gene cluster open reading frames as defined above with the polypeptide encoded by one or more safracin biosynthesis gene cluster open reading frames, whereby the polypeptide chemically modifies the compound.

In still another embodiment, this invention provides a method of producing a safracin or safracin analog. The method involves providing a microorganism transformed with an exogenous nucleic acid comprising a safracin gene cluster encoding polypeptides sufficient to direct the assembly of said safracin or safracin analog; culturing the bacteria under conditions permitting the biosynthesis of safracin or safracin analog; and isolating said safracin or safracin analog from said cell.

The invention is also directed to any of the precursor compounds P2, P14, analogs and derivatives thereof and their use in the combinatorial biosynthesis non-ribosomal peptides, diketopiperazine rings and safracins.

Additionally, the invention is also directed to the new safracins obtained by knock out safracin P19B, safracin P22A, safracin P22B, safracin D and safracin E, and their use as antimicrobial or antitumor agents, as well as their use in the synthesis of ecteinascidin compounds.

The invention is also directed to new safracins obtained by directed biosynthesis as defined above, and their use as antimicrobial or antitumor agents, as well as their use in the synthesis of ecteinascidin compounds. In particular the invention is directed to safracin B-ethoxy and safracin A-ethoxy and their use.

In one aspect, the present invention enables the preparation of structures related to safracins and ecteinascidins which cannot or are difficult to prepare by chemical synthesis. Another aspect is to use the knowledge to gain access to the biosynthesis of ecteinascidins in *Ecteinascidia turbinata*, for example using these sequences or parts as probes in this organism or a putative symbiont.

More fundamentally, the invention opens a broad field and gives access to ecteinascidins by genetic engineering.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Structural organization of the chromosomal DNA region cloned in pL30p cosmid. The region of *P. fluorescens* A2-2 DNA, containing the safracin gene cluster, is shown. Both, *sacABCDEFGH* and sacIJ, gene operons and the modular organization of the peptide synthetases deduced from *sacA*, *sacB* and *sacC* are illustrated. The following domains are indicated: C: condensation; T: thiolation; A: adenylation and Re: reductase. Location of other genes present in pL30p cosmid (*orf1* to *orf4*) as well as their proposed function is shown.

Fig. 2: Conserved core motifs between NRPSs. Conserved amino acid sequences in SacA, SacB and SacC proteins and their comparison with its homologous sequences from *Myxococcus xanthus* DM50415.

Figure 3. NRPS biosynthesis mechanism proposed for the formation of the Ala-Gly dipeptide. Step a*, adenylation of Ala; b*, transfer to the 4´-phosphopantetheinyl arm; c*, transfer to the waiting/elongation site; d*, adenylation of the Gly; e*, transfer to the 4´-phosphopantetheinyl arm; f*,

condensation of the elongation chain on the 4'-phosphopantetheinyl arm with the starter chain at the waiting/elongation site; g*, Ala-Gly dipeptide attached to the phosphopantetheinyl arm of SacA and h*, transfer of the elongated chain to the following waiting/elongation site.

Fig. 4: Cross-feeding experiments. A. Scheme of A2-2 DNA fragments cloned in pBBR1-MCS2 vector and products obtained in the heterologous host. B. HPLC profile of safracin production in wild type strain versus *sacF* mutant. The addition of P2 precursor to the *sacF* mutant, provided both *in trans* and synthetically, yield safracin B production. SfcA, safracin A and SfcB, safracin B.

Fig. 5: Scheme of the safracin biosynthesis mechanism and biosynthetic intermediates. Single enzymatic steps are indicated by a continuous arrow and multiple reactions steps are indicated by discontinuous arrows.

Fig. 6: Safracin gene disruptions and compounds produced. **A.** Gene disruption and precursor molecules synthesized by the mutants constructed. Gene marked with an asterisk does not belong to the safracin cluster. Inactivation of genes *orf1*, *orf2*, *orf3* and *orf4* has demonstrated to have no effect over safracin production. **B.** HPLC profile of safracin production in wild type strain and in *sacA*, *sacI* and *sacJ* mutants. Structure of the different molecules obtained is shown.

Fig. 7: Structure of the different molecules obtained by gene disruption. Inactivation of SacJ protein (a) yields P22B, P22A and P19 molecules, whereas gene disruption of *sacI* (b), produces only P19 compound. The *sacI* disruption, together with the *sacJ* reconstructed expression, produces two new safracins: safracin D (possible precursor for ET-729 hemi-synthesis) and safracin E (c).

Fig. 8: Addition of specific designed 'unnatural' precursors (P3). Chemical structure of the two molecules obtained by addition of P3 compound to the *sacF* mutant.

Fig. 9: Scheme of the gene disruption event through simple recombination, using an homologous DNA fragment cloned into pK18:MOB (an integrative plasmid in *Pseudomonas*).

DETAILED DESCRIPTION OF THE INVENTION

Non ribosomal peptide synthetases (NRPS) are enzymes responsible for the biosynthesis of a family of compounds that include a large number of structurally and functionally diverse natural products. For example, peptides with biological activities provide the structural backbone for compounds that exhibit a variety of biological activities such as, antibiotics, antiviral, antitumor, and immunosuppressive agents (Zuber et al. *Biotechnology of Antibiotics* 1997 (W. Strohl, ed.), 187-216 Marcel dekker, Inc., N.Y; Marahiel et al. *Chem. Rev.* 1997, 97, 2651-2673).

Although structurally diverse, most of these biologically active peptides share a common mechanistic scheme of biosynthesis. According to this model, peptide bond formation takes place on multienzymes designated peptides synthetases, on which amino acid substrates are activated by ATP hydrolysis to the corresponding adenylate. This unstable intermediate is subsequently transferred to another site of the multienzymes where it is bound as a thioester to the cysteamine group of an enzyme-bound 4′-phosphopantetheninyl (4′-PP) cofactor. At this stage, the thiol-activated substrates can undergo modifications such as epimerisation or N-methylation. Thioesterified substrate amino acids are then integrated into the peptide product through a step-by-step elongation by a series of

transpeptidation reactions. With this template arrangement in peptide synthetases, the modules seem to operate independently of one another, but they act in concert to catalyse the formation of successive peptide bonds (Stachelhaus et al. *Science* 1995, 269, 69-72; Stachelhaus et al. *Chem. Biol.* 1996, 3, 913-921). The general scheme for non-ribosomal peptide biosynthesis has been widely reviewed (Marahiel et al. *Chem. Rev.* 1997, 97, 2651-2673; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48; Moffit and Neilan, *FEMS Microbiol. Letters* 2000, 191, 159-167).

A large number of bacterial operons and fungal genes encoding peptide synthetases have recently been cloned, sequenced and partially characterized, providing valuables insights into their molecule architecture (Marahiel, *Chem and Biol.* 1997, 4, 561-567). Different cloning strategies were used, including probing of expression libraries by antibodies raised against peptide synthetases, complementation of deficient mutants, and the use of designed oligonucleotides derived from amino acid sequences of peptide synthetase fragments.

Analysis of the primary structure of these genes revealed the presence of distinct homologous domains of about 600 amino acids. This specific functional domains consist of at least six highly conserved core sequences of about three to eight amino acids in length, whose order and location within all known domains are very similar (Küsard and Marahiel, *Peptide Research* 1994, 7, 238-241). The used of degenerated oligonucleotides derived from the conserved cores opens the possibility of identifying and cloning peptide synthetases from genomic DNA, by using the polymerase chain reaction (PCR) technology (Küsard and Marahiel, *Peptide Research* 1994, 7, 238-241; Borchert et al. *FEMS Microbiol Letters* 1992, 92,175-180).

The structure of safracin suggests that this compound is synthesized

by a NRPS mechanism. The cloning and expression of the non-ribosomal peptide synthetases and the associated tailoring enzymes from *Pseudomonas fluorescens* A2-2 safracin cluster would allow production of unlimited amounts of safracin. In addition, the cloned genes could be used for combinatorial creation of novel safracin analogs that could exhibit improved properties and that could be used in the hemi-synthesis of new ecteinascidins. Moreover, cloning and expressing the safracin gene cluster in heterologous systems or the combination of safracin gene cluster with other NRPS genes could result in the creation of novel drugs with improved activities.

The present invention provides, in particular, the DNA sequence encoding NRPS responsible for biosynthesis of safracin, i.e., safracin synthetases. We have characterized a 26,705 bp region (SEQ ID NO:1) from *Pseudomonas fluorescens* A2-2 genome, cloned in pL30P cosmid and demonstrated, by knockout experiments and heterologous expression, that this region is responsible for the safracin biosynthesis. We expressed the pL30P cosmid in two strains of *Pseudomonas* sp., which do not produce safracin, and the result was a production of safracin A and B at levels of a 22%, for *P. fluorescens* (CECT 378), and 2%, for *P. aeruginosa* (CECT 110), in comparison with *P. fluorescens* A2-2 production. The predicted amino acids sequences of the various peptides encoded by this DNA sequence is shown in SEQ ID NO:2 through SEQ ID NO:15 respectively.

The gene cluster for safracin biosynthesis derived from *P. fluorescens* A2-2, is characterized by the presence of several open reading frames (ORF) that are organized in two divergent operons (**Fig. 1**), an eight genes operon (*sacABCDEFGH*) and a two genes operon (*sacIJ*), preceded by well-conserved putative promoters regions that overlap. The safracin biosynthesis gene cluster is present in only one copy in *P. fluorescens* A2-2 genome.

Our results indicate that the eight genes operon would be responsible for the safracin skeleton biosynthesis and the two genes operon would be responsible for the final tailoring of safracins.

In the s*acABCDEFGH* operon, the deduced amino acid sequences encoded by *sacA*, *sacB* and *sacC* strongly resemble gene products of NRPSs. Within the deduced amino acid sequences of SacA, SacB and SacC, one peptide synthetase module was identified on each of the ORFs.

The first surprising feature of the safracin NRPS proteins is that from the known active sites and core regions of peptide synthetases (Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48), the first core is poorly conserved in all three peptide synthetases, SacA, SacB and SacC (**Fig. 2**). The other five core regions are well conserved in the three safracin NRPSs genes. The biological significance of the first core (LKAGA) is unknown, but the SGT(ST)TGxPKG (Gocht and Marahiel, *J. Bacteriol.* 1994, 176, 2654-266; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48), the TGD (Gocht and Marahiel, *J. Bacteriol.* 1994, 176, 2654-2662; Konz and Marahiel, 1999) and the KIRGxRIEL (Pavela-Vrancic et al. *J. Biol. Chem* 1994, 269, 14962-14966; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48) core sequences could be assigned to ATP binding and hydrolysis. The serine residue of the core sequence LGGxS could be shown to be the site of thioester formation (D´Souza et al., *J. Bacteriol.* 1993, 175, 3502-3510; Vollenbroich et al., *FEBS Lett.* 1993, 325(3), 220-4; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48) and 4´-phosphopantetheine binding (Stein et al. *FEBS Lett.* 1994, 340, 39-44; Konz and Marahiel, *Chem. and Biol.* 1999, 6, R39-R48). These findings, together with the fact that safracin seems to be synthesized from amino acids, supports the hypothesis that non-ribosomal peptide bond formation via the thiotemplate mechanism is involved in the biosynthetic pathway of safracin and that

sacA, *sacB* and *sacC* encode the corresponding peptide synthetases. According to this mechanism, amino acids are activated as aminoacyl-adenylates by ATP hydrolysis and subsequently covalently bound to the enzyme via carboxyl-thioester linkages. Then, in further steps, transpeptidation and peptide bond formation occurs.

Secondly, it is striking that our sequence data clearly shows that the colinearity rule, according to which the order of the amino acid binding modules along the chromosome parallels the order of the amino acids in the peptide, does not hold for the safracin synthetase system. According to the sequence database homologies and safracin and saframycin structures homologies, SacA would be responsible for the recognition and activation of the Gly residue and SacB and SacC would be responsible for the recognition and activation of the two L-Tyr derivatives that are incorporated into the safracin skeleton, while the putative Ala-NRPS gene would be missing in the safracin gene cluster. In a few nonribosomal peptide synthetases gene clusters, such as in the pristamycin (Crecy-Lagard et al, *J. of Bacteriol.* 1997, 179(3), 705-713) and in the phosphinothricin tripeptide (Schwartz et al. *Appl Environ Microbiol* 1996, 62, 570-577) biosynthesis pathways, the first NRPS is not juxtaposed with the second NRPS gene. In concrete, in the pristamycin biosynthetic pathway the first structural gene (*snbA*) and the second structural gene (*snbC*) are 130kb apart. This is not the case for the safracin gene cluster where the results of the heterologous expression with the pL30P cosmid clearly demonstrates that there is no NRPS gene missing since there is heterologous safracin production.

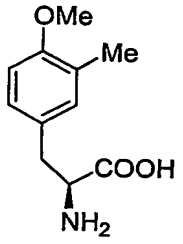
Thirdly, even though the question about the mechanism by which the dipeptide Ala-Gly is formed remains open, the presence in *sacA* of an extra C domain at the amino terminus of the first NRPS gene, suggests the possibility of a bifunctional adenylation activity by this gene. We

propose that the Ala would be first charged on the phosphopantetheinyl arm of SacA **(Fig. 3 a* and b*)** before being transferred to a waiting position, a condensation domain, located in N-terminal of *sacA* **(Fig. 3, c*)**. The Gly adenylate would then be charged on the same phosphopantetheinyl arm **(Fig. 3, d* and e*)**, positioned to the elongation site, and elongation would occur **(Fig. 3, f*)**. The arm of the first module would at this stage be charged with a Ala-Gly dipeptide **(Fig. 3, g*)**. We proposed that the dipeptide would then be transferred on a waiting position in the second phosphopantetheinyl arm **(Fig. 3, h*)**, located in SacB, to continue the synthesis of the safracin tetrapeptide basic skeleton. An alternative biosynthesis mechanism could be the direct incorporation of a dipeptide Ala-Gly into SacA. In this case, the dipeptide could be originated from the activity of highly active peptidyl transferase ribozyme family (Sun et al, *Chem. and Biol.* 2002, 9, 619-626) or from the activity of bacterial proteolysis.

And fourthly, although in most of the prokaryotic peptide synthetases the thioesterase moiety, which appears to be responsible for the release of the mature peptide chain from the enzyme, is fused to the C-terminal end of the last amino acid binding module (Marahiel et al. *Chem. Rev.* 1997, 97, 2651-2673), in the case of safracin synthetases, the TE domain is missing. Probably, in the safracin synthesis after the last elongation step, the tetrapeptide could be released by an alternative strategy for peptide-chain termination that also occurs in the saframycin synthesis (Pospiech et al. *Microbiol.* 1996, 142, 741-746). This particular termination strategy is catalysed by a reductase domain at the carboxy-terminal end of the SacC peptide synthetase which catalyses the reductive cleavage of the associated T-domain-tethered acyl group, releasing a linear aldehyde.

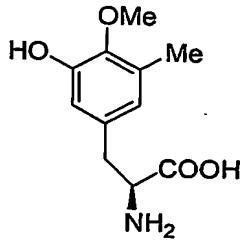
Our cross feeding experiments indicate that the last two amino acids incorporated into the safracin molecule are two L-Tyr derivatives called P2

(3-hydroxy-5-methyl-*O*-methyltyrosine) (**Fig. 4, 5**), instead of two L-Tyr as it is proposed to occur in saframycin synthesis. First, the products of two genes (*sacF* and *sacG*), similar to bacterial methyltransferases, have shown to be involved in the *O*-, *C*-methylation of L-Tyr to produce P14 (3-methyl-*O*-methyltyrosine), precursor of P2. A possible mechanism could envisage that the *O*-methylation occurs first and then the *C*-methylation of the amino acid derivative is produced. Secondly, P2, the substrate for the peptide synthetases SacB and SacC, is formed by the hydroxylation of P14 by SacD (**Fig. 4, 5**).



$C_{11}H_{15}NO_3$
Exact Mass: 209,11
Mol. Wt.: 209,24
C, 63,14; H, 7,23; N, 6,69; O, 22,94

P-14



$C_{11}H_{15}NO_4$
Exact Mass: 225,10
Mol. Wt.: 225,24
C, 58,66; H, 6,71; N, 6,22; O, 28,41

P-2

Apart from the safracin biosynthetic genes, in the *sacABCDEFGH* operon there are also found two genes, *sacE* and *sacH*, involved in an unknown function and in the safracin resistance mechanism, respectively. We have demonstrated that *sacH* gene codes for a protein that when is heterologous expressed, in different *Pseudomonas* strains, a highly increase of the safracin B resistance is produced. SacH is a putative transmembrane protein, that transforms the C_{21}–OH group of safracin B into a C_{21}-H group, to produce safracin A, a compound with less antibiotic and antitumoral activity. Finally, even though still is unknown about the putative function of SacE, homologous of this gene have been found close

to various secondary metabolites biosynthetic gene clusters in some microorganisms genomes, suggesting a conserved function of this genes in secondary metabolite formation or regulation.

In the *sacIJ* operon, the deduced amino acid sequences encoded by *sacI* and *sacJ* strongly resemble gene products of methyltransferase and hydroxylase/monoxygenase, respectively. Our data reveals that SacI is the enzyme responsible for the *N*-methylation present in the safracin structure, and that SacJ is the protein which makes an additional hydroxylation on one of the L-Tyr derivative incorporated into the tetrapeptide to produce the quinone structure present in all safracin molecules. *N*-Methylation is one of the modifications of nonribosomally synthesized peptides that significantly contributes to their biological activity. Except for saframycin (Pospiech et al. *Microbiol.* 1996, 142, 741-746), that is produced by bacteria and is *N*-methylated, all the *N*-methylated nonribosomal peptides known are produced by fungi or actinomycetes and, in most of the cases, the responsible for the *N*-methylation is a domain which reside in the nonribosomal peptide synthetase.

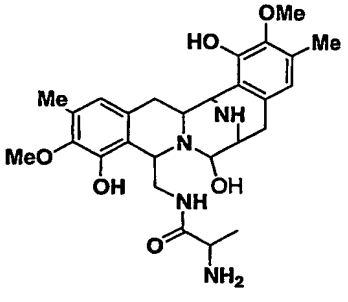
Table I. Summary of safracin biosynthetic and resistance genes identified in cosmid pL30P.

ORF name	*Protein* name	Proposed function	Position start-stop bp	Amino acids	Molecular weight
sacA	SacA	Peptide synthetase	3052-6063	1004	110.4
sacB	SacB	Peptide synthetase	6068-9268	1063	117.5
sacC	SacC	Peptide synthetase	9275-13570	1432	157.3
sacD	SacD	L-Tyr derivative hidroxylase	13602-14651	350	39.2

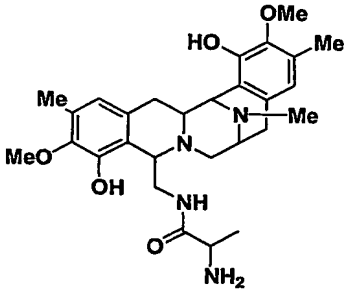
sacE	SacE	Unknown	14719-14901	61	6.7
sacF	SacF	L-Tyr derivative methylase	14962-16026	355	39.8
sacG	SacG	L-Tyr O-methylase	16115-17155	347	38.3
sacH	SacH	Resistance protein	17244-17783	180	19.6
sacI	SacI	methyl-transferase	2513-1854	220	24.2
sacJ	SacJ	monooxygenase	1861-355	509	55.3

The safracin putative synthetic pathway, with indications of the specific amino acid substrates used for each condensation reaction and the various post-condensation activities, is shown in **Fig. 5.**

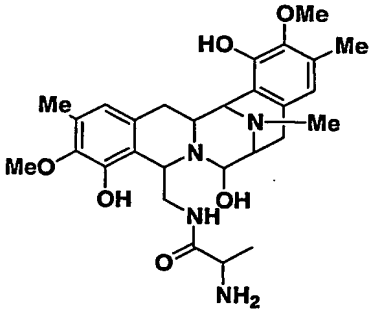
To further evaluate the role of safracin biosynthetic genes, we constructed knock out mutants of each of the genes of the safracin cluster (**Fig. 6**). The disruption of the NRPSs genes (*sacA*, *sacB* and *sacC*) as well as *sacD*, *sacF* and *sacG*, resulted in safracin and P2 non producing mutants. Our results indicate that the genes from *sacA* to *sacH* are part of the same genetic operon. As a consequence of the *sacI* and *sacJ* gene disruptions three new molecules were originated, P19B, P22A and P22B (**Fig. 6**).



P-19B



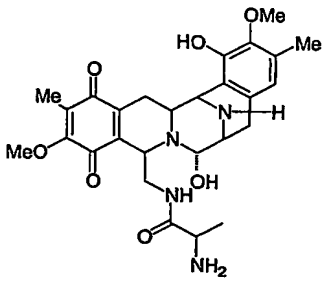
P22A



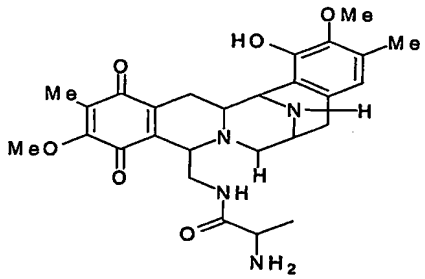
P22B

The production of P22A and P22B (**Fig. 7a***) by *sacJ* mutant demonstrated that the role of the SacJ protein is to produce the additional hydroxylation of the left L-Tyr derivatives amino acid of the safracin, the one involved in the quinone ring. The production of P19B (**Fig. 7b***) by *sacI* mutant, a safracin like molecule where the *N*-methylation and the quinone ring are missing, confirms that SacI is the N-methyltransferase enzyme and suggests that *sacIJ* is a transcriptional operon. The production of P19B also by *sacJ* mutant (**Fig. 7a***) suggests that probably the N-methylation occurs after the quinone ring has been formed. Even though these new structures have no interesting antimicrobial activity on *B. subtilis* or no high citotoxic activity on cancer cells, they can serve as interesting new precursors for the hemisynthesis of new active molecules. As far as structure activity is concerned, the observation that P19B, P22A and P22B appear to loose their activity, suggests that the lost of the quinone ring from the safracin structure is directly related with the lost of activity of the safracin family molecules.

The disruption of *sacI* gene with the reconstitution of the *sacJ* gene expression resulted in the production of two new safracins. The two antibiotics produced, at levels of production as high as the levels of safracin A/safracin B production in the wild type strain, have been named as safracin D and safracin E (**Fig. 7c***).



SAFRACINA D



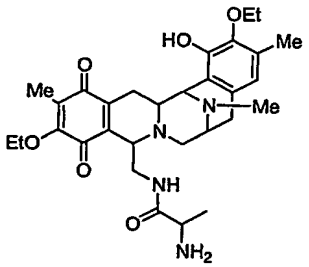
SAFRACINA E

The safracin D and safracin E are safracin B and safracin A like

molecules, respectively, where the N-methylation is missing. Both, safracin D and safracin E have been shown to possess the same antibacterial and antitumoral activities as safracin B and safracin A, respectively. Apart from its high activities properties, antibacterial and antitumoral, safracin D could be used in the hemi-synthesis of the ecteinascidin ET-729, a potent antitumoral agent, as well as in the hemi-synthesis of new ecteinascidins.

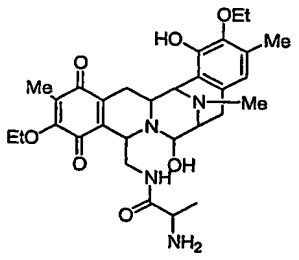
A question arises concerning the role of the aminopeptidase-like protein coded by a gene located at 3´site of the safracin operon. The insertional inactivation of *orf1* (PM-S1-14) showed no effect on safracin A/safracin B production. Because of its functionality properties it remains unclear if this protein could play some role in the safracin metabolism. The other genes present in the pL30P cosmid (*orf2* to *orf4*) will have to be studied in more detail.

Another aspect of the invention is that provides the tools necessary for the production of new specific designed "unnatural" molecules. The addition of a specific modified P2 derivative precursor named P3, a 3-hydroxy-5-methyl-*O*-methyltyrosine, to the *sacE* mutant yields two "unnatural" safracins that incorporated this specific modified precursor, safracin A(OEt) and safracin B(OEt) (**Fig. 8**).



$C_{30}H_{40}N_4O_8$
Exact Mass: 552,29
Mol. Wt.: 552,66
C, 65,20; H, 7,30; N, 10,14; O, 17,37

safracin A(OEt)



$C_{30}H_{40}N_4O_7$
Exact Mass: 568,29
Mol. Wt.: 568,66
C, 63,36; H, 7,09; N, 9,85; O, 19,69

safracin B(OEt)

The two new safracins are potent antibiotic and antitumoral compounds. The biological activities of safracin A(OEt) and Safracin B(OEt) are as potent as the activities of safracin A and safracin B, respectively. These new safracins could be the source for new potent antitumoral agents, as well as a source of molecules for the hemi-synthesis of new ecteinascidins.

In addition, the genes involved in safracin synthesis could be combined with other non ribosomal peptide synthetases genes to result in the creation of novel "unnatural" drugs and analogs with improved activities.

EXAMPLES

Example 1: Extraction of nucleic acid molecules from *Pseudomonas fluorescens* A2-2

Bacterial strains

Strains of *Pseudomonas* sp. were grown at 27°C in Luria-Bertani (LB) broth (Ausubel *et al.* 1995, J. Wiley and Sons, New York, N.Y). *E. coli* strains were grown at 37°C in LB medium. Antibiotics were used at the following concentrations: ampicillin (50 μg/ml), tetracycline (20 μg/ml) and kanamycin (50 μg/ml).

Table II. Strains used in this invention.

Code	Genotype
PM-S1-001	*P. fluorescens* A2-2 wild type
PM-S1-002	*sacA*-
PM-S1-003	*sacB*-
PM-S1-004	*sacC*-
PM-S1-005	*sacJ*-
PM-S1-006	*sacI*-
PM-S1-007	*sacI*- with *sacJ* expression reconstitution
PM-S1-008	*sacF*-
PM-S1-009	*sacG*-
PM-S1- 010	*sacD*-
PM-S1- 014	*orf1*-
PM-S1- 015	A2-2 + pLAFR3
PM-S1- 016	A2-2 + pL30p
PM-19- 001	*P. fluorescens* CECT378 + pLAFR3
PM-19- 002	*P. fluorescens* CECT378 + pL30p
PM-19- 003	*P. fluorescens* CECT378 + pBBR1-MCS2
PM-19- 004	*P. fluorescens* CECT378 + pB5H83
PM-19- 005	*P. fluorescens* CECT378 + pB7983
PM-19- 006	*P. fluorescens* CECT378 + pBHPT3
PM-16- 001	*P. aeruginosa* CECT110 + pLAFR3
PM-16- 002	*P. aeruginosa* CECT110 + pL30p
PM-17- 003	*P. putida* ATCC12633+ pBBR1-MCS2
PM-17- 004	*P. putida* ATCC12633+ pB5H83
PM-17- 005	*P. putida* ATCC12633+ pB7983
PM-18- 003	*P. stutzeri* ATCC17588+ pBBR1-MCS2
PM-18- 004	*P. stutzeri* ATCC17588+ pB5H83
PM-18- 005	*P. stutzeri* ATCC17588+ pB7983

DNA manipulation

Unless otherwise noted, standard molecular biology techniques for *in vitro* DNA manipulations and cloning were used (Sambrook *et al.* 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

DNA extraction

Total DNA from *Pseudomonas fluorescens* A2-2 cultures was prepared as reported (Sambrook *et al.* 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Computer analysis

Sequence data were compiled and analysed using DNA-Star software package.

Example 2: Identification of NRPS genes responsible for safracin production in *Pseudomonas fluorescens* A2-2.

Primer design

Marahiel *et al.* (Marahiel *et al. Chem. Rev.* 1997, 97, 2651-2673) previously reported highly conserved core motifs of the catalytic domains of cyclic and branched peptide synthetases. Based on multiple sequence alignments of several reported peptide synthetases the conserved regions A2, A3, A5, A6, A7 and A8 of adenylation and T of thiolation modules were targeted for the degenerate primer design (Turgay and Marahiel, *Peptide Res.* 1994, 7, 238-241). The wobble positions were designed in respect to codon preferences within the selected modules and the expected high G/C content of *Pseudomonas* sp. All oligonucleotides were obtained from ISOGEN (Bioscience BV). A PCR fragment was obtained when degenerate oligonucleotides derived from the YGPTE (A5 core) and LGGXS (T core) sequences were used. These oligonucleotides were denoted PS34-YG and PS6-FF, respectively.

Table III. PCR primers designed for this study.

Primer designation and orientation	**Sequence**	**Length**
PS34-YG (forward)	5'- TAYGGNCCNACNGA -3'	14-mer
PS6-FF (reverse)	5'-TSNCCNCCNADNTCRAARAA-3'	20-mer

PCR conditions for amplification of DNA from P. fluorescens A2-2

A fragment internal to nonribosomal peptide synthetases (NRPS) was amplified using PS-34-YG and PS6-FF oligonucleotides and *P. fluorescens* A2-2 chromosomal DNA as template. Reaction buffer and Taq polymerase from Promega were used. The cycling profile performed in a Personal thermocycler (Eppendorf) consists on: 30 cycles of 1 min at 95°C, 1 min at 50°C, 2 min at 72°C. PCR products were on the expected size (750 bp aprox.) based on the location of the primers within the NRPS domains of other synthetase genes.

DNA cloning

PCR amplification fragments were cloned into pGEM-Teasy vector according to the manufacturer (Qiagen, Inc., Valencia, CA). In this way, cloned fragments are flanked by two *Eco*RI restriction sites, in order to facilitate subsequent subclonig in other plasmids (see below). Since NRPSs enzymes are modular, clones from the degenerated PCR primers represents a pool of fragments from different domains.

DNA sequencing

All sequencing was performed using primers directed against the cloning vector, with an ABI Automated sequencer (Perkin-Elmer). Cloned DNA sequences were identified using the BLAST server of the National

Center for Biotechnology Information accessed over the Internet (Altschul et al., *Nucleic Acids Res.* 1997, 25, 3389-3521). All of the sequences have signature regions for NRPSs and show high similarity in BLAST searches to bacterial NRPS showing that they are in fact of peptide origin. Moreover, a probable domain similarity search was performed using the PROSITE (European Molecular Biology Laboratory, Heidelberg, Germany) web server.

Gene disruption of Pseudomonas fluorescens A2-2

In order to analyse the function of the genes cloned, these genes were disrupted through homologous recombination (**Fig. 9**). For this purpose, recombinant plasmids (pG-PS derivatives) harbouring the NRPS gene fragment were digested with *EcoR*I restriction enzyme. The resulting fragments belonging to the gene to be mutated were cloned into the pK18mob mobilizable plasmid (Schäfer *et al. Gene* 1994, 145, 69-73), a chromosomal integrative plasmid able to replicate in *E. coli* but not in *Pseudomonas* strains. Recombinant plasmids were introduced first in *E. coli* S17-λPIR strain by transformation and then in *P. fluorescens* A2-2 through biparental conjugation (Herrero *et al, J Bacteriol* 1990, 172, 6557-6567). Different dilutions of the conjugation were plated onto LB solid medium containing ampicillim plus kanamycin and incubated overnight at 27°C. Kanamycin-resistant transconjugants, containing plasmids integrated into the genome via homologous recombination, were selected.

Biological assay (biotest) for safracin production

Strains *P. fluorescens* A2-2 and its derivatives were incubated in 50 ml baffled erlenmeyer flasks containing fermentation medium with the corresponding antibiotics. Initially, SA3 fermentation medium was used (Ikeda Y. *J. Ferment. Technol.* 1985, 63, 283-286). In order to increase the productivity of the fermentation process statistical-mathematical methods like Plackett-Burman designed was used to select nutrients and response surface optimisation techniques were tested (Hendrix C. *Chemtech* 1980,

10, 488-497) in order to determine the optimum level of each key independent variable. Experiments to improve the culture conditions like incubation temperature and agitation have also been done. Finally a highly safracin B producer medium named 16B (152 g/l of mannitol, 35g/l of G20-25 yeast, 26 g/l of $CaCO_3$, 14 g/l of ammonium sulphate, 0.18 g/l of ferric chloride, pH 6.5) was selected.

The safracin production was assay testing the capacity of inhibition a *Bacillus subtilis* solid culture by 10 µl of the supernatant of a 3 days *Pseudomonas* sp. culture incubated at 27°C (Alijah et al. *Appl Microbiol Biotechnol* 1991, 34, 749-755). *P. fluorescens* A2-2 cultures produce inhibition zones of 10-14 mm diameter while non-producing mutants did not inhibit *B. subtilis* growth. Three isolated clones had the safracin biosynthetic pathway affected. In order to confirm the results, HPLC analysis of safracin production was performed.

HPLC analysis of safracin production.

The supernatant was analysed by using HPLC Symmetry C-18. 300Å, 5 µm , 250 x 4.6 mm column (Waters) with guard-column (Symmetry C-18, 5µm 3.9 x 20 mm, Waters). An ammonium acetate buffer (10 mM, 1% Diethanolamine, pH 4.0)- acetonitrile gradient was the mobile phase. Safracin was detected by absorption at 268 nm. In **Fig. 6,** HPLC profile of safracin and safracin precursors produce by *P. fluorescens* A2-2 strain and different safracin-like structures produced by *P. fluorescens* mutants are shown.

Example 3. Cloning and sequence analysis of safracin cluster

Inverse PCR and phage library hybridisation

Southern hybridisation on mutant chromosomal DNAs verified the correct gene disruption and demonstrated that the peptide synthethase fragment cloned into pK18mob plasmid was essential for the production of safracin. Analysis of the non safracin producers mutants obtained demonstrated that all of them had a gene disruption into the same gene, *sacA*.

Inverse PCR from genomic DNA and screening of a phage library of *P. fluorescens* A2-2 genomic DNA revealed the presence of additional genes flanking *sacA* gene, probably involved in safracin biosynthesis.

The GenBank accession number for the nucleotide sequence data of the *P. fluorescens* A2-2 safracin biosynthetic cluster is AY061859.

Cosmid library construction and heterologous expression

To determine whether safracin cluster was able to confer safracin biosynthetic capability to a non producer strain, it was cloned into a wide range cosmid vector (pLAFR3, Staskawicz B. *et al. J Bacteriol* 1987, 169, 5789-5794) and conjugated to a different *Pseudomonas* sp collection strains.

To obtain a clone containing the whole cluster, a cosmid library was constructed and screened. For this purpose, chromosomal DNA was partially digested with the restriction enzyme *PstI*, the fragments were dephosphorylated and ligated into the *PstI* site of cosmid vector pLAFR3. The cosmids were packaged with Gigapack III gold packaging extracts (Stratagene) as manufacturer's recommendations. Infected cells of strain XL1-Blue were plated on LB-agar supplemented with 50 μg/ml of tetracycline. Positives clones were selected using colony hybridization with a DIG-labeled DNA fragment belonging to the 3'-end of the safracin cluster. In order to ensure the cloning of the whole cluster, a new colony hybridization with a 5'-end DNA fragment was done. Only cosmid pL30p showed multiple hybridizations with DNA probes. To confirm the accurate cloning, PCR amplification and DNA-sequencing with DNA oligonucleotides

belonging to the safracin sequence were carried out. The size of the insert of pL30P was 26,705 bp . The pL30p clone DNA was transformed into *E. coli* S17λPIR and the resulting strain were conjugated with the heterologous *Pseudomonas* sp. strains. The pL30p cosmid was introduced into *P. fluorescens* CECT378 and *P. aeruginosa* CECT110 by biparental conjugation as described above. Once a clone encoding the whole cluster was identified, it was determined whether the candidate was capable of producing safracin. Safracin production in the conjugated strains was assessed by HPLC analysis and biological assay of broth cultures supernatants as previously described.

The strain *P. fluorescens* CECT378 expressing the pL30p cosmid (PM-19-002) was able to produce safracin in considerable amounts, whereas safracin production in *P. aeruginosa* CECT110 strain expressing pL30P (PM-16-002) was 10 times less than the CECT378. Safracin production in these strains was about 22 % and 2 % of the total production in comparison with the natural producer strain.

Genes involved in the formation of safracin. Sequence analysis of sacABCDEFGH *and* sacIJ *operons*

Computer analyses of the DNA sequence of pL30P revealed 14 ORFs (**Fig. 1**). A potential ribosome binding site precedes each of the ATG start codons.

In the *sacABCDEFGH* operon, three very large ORFs, *sacA*, *sacB* and *sacC* (positions 3052 to 6063, 6080 to 9268 and 9275 to 13570 of the *P. fluorescens* A2-2 safracin sequence SEQ ID NO:1, respectively) can be read in the same direction and encode the putative safracin NRPSs: SacA (1004 amino acids, M_r 110452), SacB (1063 amino acids, M_r 117539) and SacC (1432 amino acids, M_r 157331). The three NRPSs genes contain the domains resembling amino acid activating domains of known peptide synthetases. Specifically, the amino acid activating domains from these NRPS genes are very similar to three of the four amino acid activating

domains (Gly, Tyr and Tyr) found in the *Myxococcus xanthus* saframycin NRPSs (Pospiech et al. *Microbiology* 1995, 141, 1793-803; Pospiech et al. *Microbiol.* 1996, 142, 741-746). In particular, SacA (SEQ ID NO:2) shows 33% identity with saframycin Mx1 synthetase B protein (SafB) from *M. xanthus* (NCBI accession number U24657), whereas SacB (SEQ ID NO:3) and SacC (SEQ ID NO:4) share, respectively, 39% and 41% identity with saframycin Mx1 synthetase A (SafA) from *M. xanthus* (NCBI accession number U24657). The **Fig. 2** shows a comparison among SacA, SacB y SacC and the different amino acid activating domains of saframycin NRPS.

Downstream *sacC* five small ORFs reading in the same direction as the NRPSs genes exist (**Fig.1**). The first one, *sacD* (position 13602 to 14651 of *P. fluorescens* A2-2 safracin sequence), encodes a putative protein, SacD (350 amino acids, M_r 39187; SEQ ID NO:5), with no similarities in the GeneBank DB. The next one, *sacE* (position 14719 to14901 of *P. fluorescens* A2-2 safracin sequence), encodes a small putative protein called SacE (61 amino acids, M_r 6729; (SEQ ID NO:6)), which shows some similarity with proteins of unknown function in the databases (ORF1 from *Streptomyces viridochromogenes* (NCBI accession number Y17268; 44% identity) and MbtH from *Mycobacterium tuberculosis* (NCBI accession number Z95208; 36% identity). The third ORF, *sacF* (position 14962 to 16026 of *P. fluorescens* A2-2 safracin sequence), encodes a 355-residue protein with a molecular weigh calculated of 39,834 (SEQ ID NO:7). This protein most closely resembles hydroxyneurosporene methyltransferase (CrtF) from *Chloroflexus aurantiacus* (NCBI accession number AF288602; 25% identity). The nucleotide sequence of the fourth ORF, *sacG* (position 16115 to 17155 of *P. fluorescens* A2-2 safracin sequence), predicted a gene product of 347 amino acids having a molecular mass of 38,22 kDa (SEQ ID NO:8). The protein, called SacG, is similar to bacterial O-methyltransferases, including O-dimethylpuromycin-O-methyltransferase (DmpM) from *Streptomyces anulatus* (NCBI accession number P42712; 31% identity). A computer search also shows that this protein contains the

three sequence motifs found in diverse S-adenosylmethionine-dependent methytransferases (Kagan and Clarke, *Arch. Biochem. Biophys.* 1994, 310, 417-427). The fifth gene, *sacH* (position 17244 to 17783 of *P. fluorescens A2-2* safracin sequence), encodes a putative protein SacH (180 amino acids, M_r 19632; (SEQ ID NO:9). A computer search for similarities, between the deduced amino acid sequence of SacH and other protein sequences, revealed identity with some conserved hypothetical proteins of unknown function, which contains a well conserved transmembrane motif and a dihydrofolate reductase-like active site (Conserved hypothetical protein from *Pseudomonas aeruginosa* PAO1, NCBI accession number P3469; 35% identity).

Upstream s*acABCDEFGH* operon, reading in opposite sense, a two genes operon, *sacIJ*, is located. The *sacI* gene (position 2513 to 1854) encodes a 220-amino acids protein (M_r 24219; (SEQ ID NO:10) that most closely resembles ubiquinone/manequinone methyltrasnferase from *Thermotoga maritime* (NCBI accession number AE001745; 32% identity). The *sacJ* gene (position 1861 to 335) encodes a 509-amino acid protein (SEQ ID NO:11), with a molecular mass of 55341 Da, similar to bacterial monooxygenases/hydroxylases, including putative monooxygenase from *Bacillus subtilis* (NCBI accession number Y14081; 33% identity) and *Streptomyces coelicolor* (NCBI accession number AL109972; 29% identity).

SacABCDEFGH and *sacIJ* operons are transcribed divergently and are separated by 450 bp approximately. Both operons are flanked by residual transposase fragments.

Related safracin cluster genes

A putative ORF (*orf1*; position 18322 to 19365 of *P. fluorescens* A2-2 safracin sequence) located at the 3'-end of the safracin sequence has been

found (**Fig. 1**). ORF1 protein (SEQ ID NO:12) shows similarity with aminopeptidases from the Gene Bank DataBase (peptidase M20/M25/M40 family from *Caulobacter crescentus* CB15; NCBI accession number NP422131; 30% identity). Using the strategy described in Example 2, the gene disruption of *orf1* do not affect safracin production in *P. fluorescens* A2-2.

At the 3'-end of the safracin sequence cloned in pL30p cosmid, three putative ORFs (*orf2, orf3* and *orf4*), were found. Reading in opposite direction than *sacABCDEFGH* operon, *orf2* gene (position 22885 to 21169 of SEQ ID NO:1) codes for a protein, ORF2 (SEQ ID NO:13), with similarities to *Aquifex aeolicus* HoxX sensor protein (NCBI accession number NC000918.1; 35% identity), whereas *orf3* gene (position 23730 to 23041 of SEQ ID NO:1) codes for ORF3 protein (SEQ ID NO:14) which shares 44% identity with a glycosil transferase related protein from *Xanthomonas axonopodis* pv. Citri str. 306 (NCBI accession number NP642442).

The third gene is located at the 3'-end of SEQ ID NO:1 (position 25037 to 26095). This gene, named *orf4* (position 2513 to 1854), encodes a protein, ORF4 (SEQ ID NO:15), that most closely resembles to a hypothetical isochorismatase family protein YcdL from *Escherichia coli.* (NCBI accession number P75897; 32% identity).

Presumably, these three genes would not be involve in the safracin biosynthetic pathway, however, future gene disruption of these genes will confirm this assumption.

The different DNA sequences found are listed at the end of the description.

Example 4. Functional analysis of the safracin loci and search for

possible precursors

Since the pathway for synthesis of safracin in *P. fluorescens* A2-2 is at present unknown, the inactivation of each of the genes described in Example 3 would permit fundamental studies on the mechanism of safracin biosynthesis in this strain.

In order to analyze the functionality of each particular protein in the safracin production pathway, disruption of each particular gene of the cluster, but *sacE*, was performed. All of the genetic mutants were obtained following the disruption strategy previously described.

Figure 6 is a summary of the different mutants constructed in this invention as well as a summary of the compounds produced by the mutants as a consequence of the gene disruption. In the wild type strain both safracin A and B and other compounds, P2 and P14, were clearly detected by HPLC (see **Fig. 6,WT**). The gene disruption of the *sacA* (PM-S1-002), *sacB* (PM-S1-003), *sacC* (PM-S1-004), *sacD* (PM-S1-010), *sacF* (PM-S1-008), and *sacG* (PM-S1-009), genes generated mutants that were unable to produce neither safracin A and safracin B, nor the precursor compounds with retention times beneath 15 min, P2 and P14 respectively. The structure elucidation of P14 and P2 revealed that P14 is a 3-methyl-*O*-methyl tyrosine, where as P2 is a 3-hydroxy-5-methyl-*O*-methyl tyrosine. Because of the small size of the *sacE* gene, the *sacE*$^-$ mutant was not possible to be obtained by gene disruption, but deletion of this gene is in process. The overexpression of SacE protein, *in trans*, had no effect on safracin B/A production. The *sacI*$^-$ mutants (PM-S1-006) produced P2, P14 and significant amount of a compound called P19B (**Fig. 6; Fig7b***). Structure elucidation of P19B revealed that this compound is a safracin-like molecule in which the *N*-Met and one of the OH from the quinone ring are missing. In the *sacJ*$^-$ mutants (PM-S1-005), P2, P14, P19B and two new compounds called P22A and P22B were obtained (**Fig. 6; Fig. 7a***).

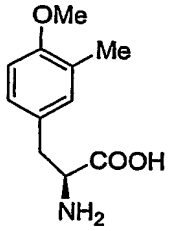
Structure elucidation of P22A and P22B revealed that they are safracin A and safracin B like molecules, respectively, without one of the -OH group from the quinone ring. The biological assay of the *sacI*- and the *sacJ*- mutants extracts revealed very low activity against *Bacillus subtilis*.

The disruption of *sacI* gene with the reconstitution of the *sacJ* gene expression resulted in a new safracins producer mutant, PM-S1-007. The two antibiotics produced, at levels of production as high as the levels of safracin A and safracin B in the wild type strain, have been named as safracin D and safracin E (**Fig. 7c***). The safracin D and safracin E are safracin B and safracin A like molecules, respectively, where the N-methylation is missing.

These results strongly suggest that i) *sacA*, *sacB* and *sacC* genes encode for the safracin NRPSs; ii) *sacD*, *sacF* and *sacG* genes are responsible for the transformation of L-Tyr into the L-Tyr derivative P2 and iii) *sacI* and *sacJ* are responsible for the tailoring modifications that convert P19 and P22 into safracin.

Characterization of Natural Precursors:

P-14



$C_{11}H_{15}NO_3$
Exact Mass: 209,11
Mol. Wt.: 209,24
C, 63,14; H, 7,23; N, 6,69; O, 22,94

Strain:

Pseudomonas fluorescens A2-2 (wild type) (PM-S1-001)

Fermentation conditions:

Seed medium YMP3 containing 1% glucose; 0.25% beef extract; 0.5% bacto-peptone; 0.25% NaCl; 0,8% CaCO3 was inoculated with 0.1% of a frozen vegetative stock of the microorganism, and incubated on a rotary shaker (250 rpm) at 27°C. After 30h of incubation, the 2% (v/v) seed culture was transferred into 2000 ml Erlenmeyer flasks containing 250 ml of the M-16B production medium, composed of 15.2 % mannitol; 3.5 % Dried brewer's yeast; 1.4 % $(NH_4)_2$ SO_4; 0.001%; $FeCl_3$; 2.6 % CO_3Ca. The temperature of the incubation was 27°C from the inoculation till 40 hours and then, 24°C to final process (71 hours). The pH was not controlled. The agitation of the rotatory shaker was 220 rpm with 5 cm eccentricity.

Isolation:

After 71 hours of incubation, 2 Erlenmeyer flasks were pooled and the 500 ml of fermentation broth was clarified by 7.500 rpm centrifugation during 15 minutes. 50 grams of the resin XAD-16 (Amberlite) were added to the supernatant and mixed during 30 minutes at room temperature. Then, the resin was recovered from the clarified broth by filtration. The resin was washed twice with distilled water and extracted with 250 ml of isopropanol (2-PrOH). The alcohol extract was dried under high vacuum till obtention of 500 mg crude extract. This crude was dissolved in methanol and purified by chromatographic column using Sephadex LH-20 and methanol as mobile phase. The P-14 compound was eluted and dried as a 15 mg yellowish solid. The purity was tested by analytical HPLC and ^1H NMR.

P-14 was also isolated in a similar way from cultures of the *sacJ*- mutant (PM-S1-005), using semipreparative HPLC as the last step in the purification process.

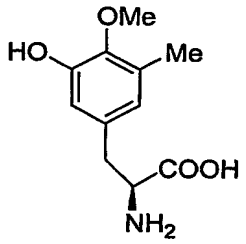
Biological activities:

NO ACTIVE

Spectroscopic data:

ESMS *m/z* 254 ($C_{11}H_{14}NO_3Na_2^+$), 232 ($C_{11}H_{15}NO_3Na^+$), 210 ($M+H^+$). ^1H RMN (300 MHz, CD_3OD): 7.07 (d, *J*=8.1 Hz, H-9), 7.06 (s, H-5), 6.84 (d, *J*=8.1 Hz, H-8), 3.79 (s, H-11), 3.72 (dd, *J*=8.7, 3.9 Hz, H-2), 3.20 (dd, *J*=14.4, 3.9 Hz, H-3a), 2.91 (dd, *J*=14.4, 8.9 Hz, H-3b), 2.16 (s, H-10). ^{13}C RMN (75 MHz, CD_3OD): 174.1 (C-1), 158.6 (C-7), 132.5 (C-5), 128.9 (C-9), 128.5 (C-4), 128.0 (C-6), 111.4 (C-8), 57.6 (C-2), 55.8 (C-11), 37.4 (C-3), 16.3 (C-10)

P-2



$C_{11}H_{15}NO_4$
Exact Mass: 225,10
Mol. Wt.: 225,24
C, 58,66; H, 6,71; N, 6,22; O, 28,41

Strain:

Pseudomonas fluorescens A2-2 (wild type) (PM-S1-001)

Fermentation conditions:

The same process than P-14

Isolation:

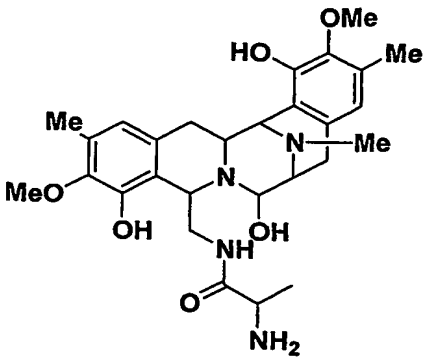
Similar procedure as the P-14, except in the Sephadex chromatography, where the fractions containing P-2 have eluted later. A semi-preparative HPLC step (Symmetry Prep C-18 column, 7.8 x 150 mm, $AcONH_4$ 10 mM pH=3/CH_3CN 95:5 held for 5 min and then gradient from 5 to 6.8 % of CH_3CN in 3 min) has been necessary to purify the P-2.
Also this compound has been isolated from the fermentation broth of the *Pseudomonas putida* ATCC12633+pB5H83 (PM-17-004) as result of heterologous expression.

Biological activities:
NO ACTIVE

Spectroscopic data:
ESMS *m/z* 226 $[M+H]^+$; ^1H RMN (CD_3OD, 300 MHz): 6.65 (d, J = 1.8 Hz, H-5), 6.59 (d, J = 1.8 Hz, H-9), 3.72 (s, H-11), 3.71 (dd, J = 9.0, 4.2 Hz, H-2), 3.16 (dd, J = 14.4, 4.2 Hz, H-3a), 2.83 (dd, J = 14.4, 9.0 Hz, H-3b), 2.22 (s, H-10); ^{13}C RMN (DMSO, 75 MHz): 170.88 (s, C-1), 150.025 (s, C-7), 144.56 (s, C-8), 132.28 (s, C-4), 130.36 (s, C-6), 121.73 (d, C-5), 115.55 (d, C-9), 59.06 (q, 7-OMe), 55.40 (d, C-2), 36.21 (t, C-3), 15.86 (q, 6-Me).

Characterization of Safracins like compounds obtained by knock out

COMPOUND P-22B



$C_{28}H_{38}N_4O_6$
Exact Mass: 526,28
Mol. Wt.: 526,62
C, 63,86; H, 7,27; N, 10,64; O, 18,23

Strain:

sac J$^-$ mutant from *P.fluorescens A2-2* (PM-S1-005)

Fermentation conditions:

50 liters of the SAM-7 medium (50 l) composed of dextrose (3.2%), mannitol (9.6%), dry brewer's yeast (2%), ammonium sulphate (1.4%), potassium secondary phosphate (0.03%), potassium chloride (0.8%), Iron (III) chloride 6-hydrtate (0.001%), L-tyrosine (0.1%), calcium carbonate (0.8%), poly- (propylene glycol) 2000 (0.05%) and antifoam ASSAF 1000 (0.2%) was poured into a jar-fermentor (Bioengineering LP-351) with 75 l total capacity and, after sterilization, sterile antibiotics (amplicillin 0.05 g/l and kanamycin 0.05 g/l) were added. Then, it was inoculated with seed culture (2%) of the mutant strain PM-S1-005. The fermentation was carried out during 71 h. under aerated and agitated conditions (1.0 l/l/min and 500 rpm). The temperature was controlled from 27°C (from the inoculation till 24 hours) to 25°C (from 24h to final process). The pH

was controlled at pH 6.0 by automatic feeding of diluted sulphuric acid from 22 hours to final process.

Isolation

The whole broth was clarified (Sharples centrifuge). The pH of the clarified broth was adjusted to pH 9.0 by addition of NaOH 10% and extracted with 25 litres of ethyl acetate. After 20' mixing, the two phases were separated. The organic phase was frozen overnight and then, filtered for removing ice and evaporated to a greasy dark green extract (65.8 g).
This extract was mixed with 500 ml hexane (250 ml two times) and filtered for removing hexane soluble impurities. The remaining solid, after drying, gave a 27.4 g of a dry green-beige extract.
This new extract was dissolved in methanol and purified by a Sephadex LH-20 chromatography (using methanol as mobile solvent) and the safracins-like compounds were eluted in the central fractions (*Analyzed on TLC conditions: Silica normal phase, mobile phase: EtOAc:MeOH 5:3. Aprox. Rf valor: 0.3 for P-22B, 0.25 P-22A and 0.1 for P-19).*
The pooled fractions, (7,6g) containing the three safracin-like compound were purified by a Silica column using a mixture of EtOAc:MeOH from 50:1 to 0:1. and other chromatographic system (isocratic $CHCl_3$:MeOH:H_2O:AcOH 50:45:5:0.1). Compounds P22-A, P22-B and P19-B were purified by reversed-phase HPLC (SymmetryPrep C-18 column 150 x 7.8 mm, 4 mL/min, mobile phase: 5 min MeOH:H_2O (0.02 % TFA) 5:95 and gradient from MeOH:H_2O (0.02 % TFA) 5:95 to MeOH 100 % in 30 min).

Biological activities of safracin P-22B

Primary Screening		Cells Lines (Mol/L)																
		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix		
		PC-3	LNcap	SK-OV-3	IGROV	IGROVET	BT-549	SK-MEL-28	HMEC	A-549	K-562	PANC-1	HT29	LOVO	LOVO-DOX	HeLa	HELA-APL	
PM-	GI50	4.58E-06	3.08E-07		8.49E-07	3.02E-06	8.24E-07	5.20E-07		4.71E-06	1.13E-07	4.77E-06	1.01E-05	2.54E-05	6.93E-06	7.61E-07	4.65E-07	
	TGI	8.52E-06	6.09E-07		2.30E-06	7.04E-06	2.28E-06	9.99E-07		8.83E-06	4.67E-07	1.17E-05	2.76E-06	6.84E-06	1.90E-05	1.83E-06	9.32E-07	
23-01	LC50	1.62E-05	1.20E-06		1.21E-05	1.65E-05	8.85E-06	2.01E-06		1.65E-05	1.84E-06	> 1.90E-05	1.86E-05	1.84E-05	> 1.90E-05	7.42E-06	1.86E-06	

Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): 10 mm inhibition zone

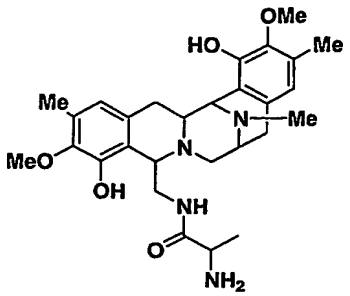
Spectroscopic data:

HRFABMS *m/z* 509.275351 $[M-H_2O+H]^+$ (calcd for $C_{28}H_{37}N_4O_5$ 509.276396 Δ 1.0 mmu); LRFABMS using m-NBA as matrix *m/z* (rel intensity) 509 $[M-H_2O+H]^+$ (5), 460 (2.7), 391 (3).

^1H NMR (CD_3OD, 500 MHz): 6.70 (s, H-15), 6.52 (s, H-5), 4.72 (bs, H-11), 4.66 (d, *J* = 2.0 Hz, H-21), 4.62 (dd, *J* = 8.4, 3.7 Hz, H-1), 3.98 (bd, *J* = 7.6 Hz, H-13), 3.74 (s, 7-OMe), 3.71 (s, 17-OMe), 3.63 (m, overlapped signal, H-25), 3.62 (m, overlapped signal, H-3), 3.30 (m, H-22a), 3.29 (m, H-14a), 3.18 (d, *J* = 18.6 Hz, H-14b), 2.90 (m, H-4a), 2.88 (m, H-22b), 2.76 (s, 12-NMe), 2.30 (s, 16-Me), 2.22 (m, H-4b), 1.16 (d, *J* = 7.4 Hz, H-26);

^{13}C NMR (CD_3OD, 125 MHz): 170.75 (s, C-24), 149.24 (s, C-18), 147.54 (s, C-8), 145.95 (s, C-7), 145.82 (s, C17), 133.93 (s, C-16), 132.31 (s, C-9), 131.30 (s, C-6), 128.95 (s, C-20), 121.93 (d, C-15), 121.76 (d, C-5), 121.44 (s, C-10), 112.45 (s, C-19), 92.87 (d, C-21), 60.86 (q, 7-OMe), 60.76 (q, 17-OMe), 59.39 (d, C-11), 57.96 (d, C-13), 55.51 (d, C-1), 54.29 (d, C-3), 50.08 (d, C-25), 45.55 (t, C-22), 40.43 (q, 12-NMe), 32.56 (t, C-4), 25.84 (t, C-14), 17.20 (q, C-26), 16.00 (q, 16-Me), 15.81 (q, 6-Me).

COMPOUND P-22A



Strain:

The same as for P-22B

Fermentation conditions:

The same as for P-22B

Isolation:

The same as for P-22B

Biological activities of safracin P-22A

Antitumor activities

Cells Lines (Mol/L)																			
Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon				Cervix	
		DU145	LNCaP	SK-OV-3	IGROV	IGROVER	SK-BR3	SKMEL-28	HMEC1	A549	K562	PANC1	HTH-1	LOVO	LOVO-DOX		HELA	HELA-APL
Safracin P-22A	GI50	> 1.96E-05	4.19E-06		7.74E-06	1.30E-05	1.27E-05	5.93E-06		> 1.96E-05	3.15E-06	> 1.96E-05	1.26E-05	> 1.96E-05	> 1.96E-05		8.75E-06	7.66E-06
	TGI	> 1.96E-05	9.26E-06		1.96E-05	> 1.96E-05	> 1.96E-05	1.33E-05		> 1.96E-05	7.93E-06	> 1.96E-05	> 1.96E-05	> 1.96E-05	> 1.96E-05		> 1.96E-05	1.96E-05
	LC50	> 1.96E-05	> 1.96E-05		> 1.96E-05	> 1.96E-05	> 1.96E-05	> 1.96E-05		> 1.96E-05	1.95E-05	> 1.96E-05	> 1.96E-05	> 1.96E-05	> 1.96E-05		> 1.96E-05	> 1.96E-05

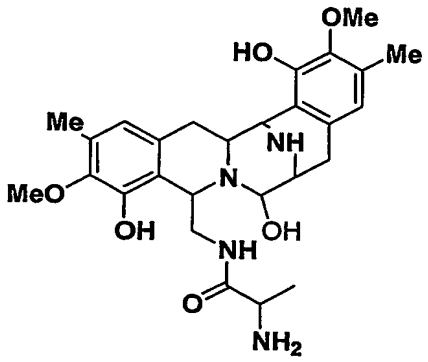
Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): NO ACTIVE

Spectroscopic data:

HRFABMS *m/z* 511.290345 $[M+H]^+$ (calcd for $C_{28}H_{39}N_4O_5$ 511.292046 Δ 1.7 mmu); LRFABMS using m-NBA as matrix *m/z* (rel intensity) 511 $[M+H]^+$ (61), 409 (25), 391 (4); ^1H NMR (CD_3OD, 500 MHz): 6.68 (s, H-15), 6.44 (s, H-5), 3.71 (s, 7-OMe), 3.67 (s, 17-OMe), 2.72 (s, 12-NMe), 2.28 (s, 16-Me), 2.20 (s, 6-Me), 0.87 (d, J = 7.1 Hz, H-26);

COMPOUND P-19B



Strain:

The same as for P-22B

Fermentation conditions:

The same as for P-22B

Isolation

The same as for P-22B

Biological activities of safracin P-19B

Antitumor activities

Primary Screening		Cells Lines (Mol/L)																	
		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon				Cervix	
		DU-145	LNCaP	SK-OV-3	IGROV1	OVCAR-3	SK-BR-3	MDA-MB-435	HUVEC	A549	HL-60(TB)	PANC-1	HT29	LOVO	LOVO-DOX	KB-A1	HELA-S3
safracin P-19B	GI50	1.70E-05	3.30E-05		5.42E-06	8.74E-06	7.08E-06	7.80E-06		> 1.95E-05	2.39E-06	1.91E-05	1.55E-05	> 1.95E-05	1.44E-05	6.73E-06	4.80E-06
	TGI	> 1.95E-05	8.05E-05		1.48E-05	> 1.95E-05	1.92E-05	> 1.95E-05		> 1.95E-05	5.77E-06	> 1.95E-05	> 1.95E-05	> 1.95E-05	> 1.95E-05	1.51E-05	1.00E-05
23-OCT-02	LC50	> 1.95E-05	1.67E-05		> 1.95E-05	> 1.95E-05	> 1.95E-05	> 1.95E-05		> 1.95E-05	1.40E-05	> 1.95E-05	> 1.95E-05	> 1.95E-05	> 1.95E-05	> 1.95E-05	1.95E-05

Antimicrobial activity: On solid medium

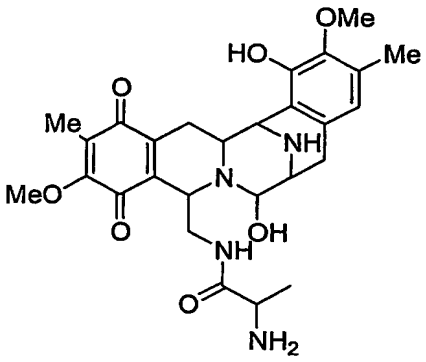
Bacillus subtilis. 10µg/disk (6mm diameter): NO ACTIVE

Spectroscopic data:

HRFABMS *m/z* 495.260410 $[M\text{-}H_2O+H]^+$ (calcd for $C_{27}H_{35}N_4O_5$ 495.260746 Δ 0.3 mmu); LRFABMS using m-NBA as matrix *m/z* (rel intensity) 495 $[M\text{-}H_2O+H]^+$ (13), 460 (3), 391 (2); ^1H NMR (CD_3OD, 500 MHz): 6.67 (s, H-15), 6.5 (s, H-5), 3.73 (s, 7-OMe), 3.71 (s, 17-OMe), 2.29 (s, 16-Me), 2.24 (s, 6-Me), 1.13 (d, J = 7.1 Hz, H-26);

New Safracin compounds obtained by knock out

SAFRACIN D



$C_{27}H_{34}N_4O_7$
Exact Mass: 526,24
Mol. Wt.: 526,58
C, 61,58; H, 6,51; N, 10,64; O, 21,27

Strain:

sac I- with *sacJ* expression reconstitution from *P.fluorescens* A2-2 (PM-S1-007)

Fermentation conditions:

50 litres of the SAM-7 medium (50 l) composed of dextrose (3.2%), mannitol (9.6%), dry brewer's yeast (2%), ammonium sulphate (1.4%), potassium secondary phosphate (0.03%), potassium chloride (0.8%), Iron (III) chloride 6-hydrtate (0.001%), L-tyrosine (0.1%), calcium carbonate (0.8%), poly- (propylene glycol) 2000 (0.05%) and antifoam ASSAF 1000 (0.2%) was poured into a jar-fermentor (Bioengineering LP-351) with 75 l total capacity and, after sterilization, sterile antibiotics (amplicillin 0.05 g/l and kanamycin 0.05 g/l) were added. Then, it was inoculated with seed culture (2%) of the mutant strain PM-S1-007. The fermentation was carried out during 89 h. under aerated and agitated conditions (1.0

1/1/min and 500 rpm). The temperature was controlled from 27°C (from the inoculation till 24 hours) to 25°C (from 24h to final process). The pH was controlled at pH 6.0 by automatic feeding of diluted sulphuric acid from 27 hours to final process.

Isolation:

The cultured medium (45 l) thus obtained was, after removal of cells by centrifugation, adjusted to pH 9.5 with diluted sodium hydroxide, extracted with 25 liter of ethyl acetate twice. The mixture was carried out into an agitated-vessel at room temperature for 20 minutes. The two phases were separated by a liquid-liquid centrifuge. The organic phases were frozen at –20°C and filtered for removing ice and evaporated until obtention of a 35g. oil-dark-crude extract. After a 5 l. hexane triturating, the extract (12.6g) was purified by a flash-chromatographic column (5.5 cm diameter, 20 cm length) on silica-normal phase, mobile phase: Ethyl acetate: MeOH: 1 L of each 1:0; 20:1; 10:1; 5:1 and 7:3. 250 ml- fractions were eluted and pooled depending of the TLC (Silica-Normal, EtOAc:MeOH 5:2, Safracin D Rf 0.2, safracin E 0.05). The fraction containing impure safracin D and E was evaporated under high vacuum (2.2 g). An additional purification step was necessary to separate D and E on similar conditions (EtOAc:MeOH from 1:0 to 5:1), from this, the fractions containing safracin D and E are separate and evaporated and further purification by Sephadex LH-20 column chromatography eluted with methanol.

The safracins D and E obtained were independent precipitated from CH_2Cl_2 (80 ml) and Hexane (1500 ml) as a green/yellowish-dried solid (800 mg safracin D) and (250 mg safracin E).

Biological activities Safracin D

Antitumor screening:

Cells Lines (Mol/L)																		
Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix		
		DU-145	LNCaP	SK-OV3	IGROV	IGROV-ET	SK-BR3	SK-MEL-28	HMEC1	A549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA/APL	
PM - Fernando de la Calle020	GI50	5.22E-06	1.54E-06		2.68E-06	1.33E-06	4.71E-06	3.51E-06		6.04E-06	6.04E-07	4.77E-06	4.33E-06	6.99E-06	4.75E-06	3.76E-06	2.28E-06	
	TGI	9.99E-06	4.12E-06		6.02E-06	3.34E-06	7.82E-06	6.21E-06		1.07E-05	1.16E-06	1.10E-05	1.79E-05	1.82E-05	8.85E-06	6.68E-06	5.24E-06	
19-AUG-02	LC50	1.90E-05	9.78E-06		1.35E-05	9.15E-06	1.30E-05	1.10E-05		1.89E-05	3.78E-06	> 1.90E-05	> 1.90E-05	> 1.90E-05	1.85E-05	1.19E-05	1.21E-05	

Secondary Evaluation (Mol/L)										
Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton			
		PROTEIN	DNA	RNA	NUCLEOSOLES	GEL	ACTIN	TUBULIN	TELOMERASE	
PM - Fernando de la Calle020 20-AUG-02	IC50	1.90E-05	1.52E-05	3.80E-06	2.85E-06	6.65E-06				

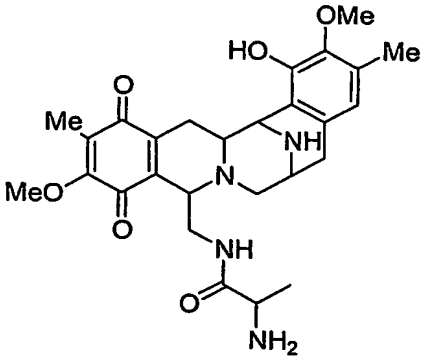
Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): Inhibition zone: 15 mm diameter

Spectroscopic data

ESMS: *m/z* 509 $[M-H_2O+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz): 6.50 (s, C-15), 4.02 (s, OMe), 3.73 (s, OMe), 2.22 (s, Me), 1.85 (s, Me), 0.80 (d, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): 186.51, 181.15, 175.83, 156.59, 145.09, 142.59, 140.78, 137.84, 131.20, 129.01, 126.88, 121.57 (2 x C), 82.59, 60.92, 60.69, 53.12, 21.40, 50.68, 50.22, 48.68, 40.57, 29.60, 25.01, 21.46, 15.64, 8.44.

SAFRACIN E



$C_{27}H_{34}N_4O_6$
Exact Mass: 510,25
Mol. Wt.: 510,58
C, 63,51; H, 6,71; N, 10,97; O, 18,80

Strain:

The same than safracin D

Fermentation conditions:

The same batch as safracin D

Isolation:

See safracin D conditions

Biological activities Safracin E

Antitumor screening:

Cells Lines (Mol/L)																		
Primary Screening		Prostate			Ovary		Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon				Cervix	
		DU145	LNaP	SK-OV-3	IGROV	IGROV1	SK-BR3	SK-MEL-28	HMEC1	A549	K-562	PANC1	HT29	LOVO	LOVO-DOX	HELA	HELA-R
PM - Fernando de la Calle020	GI50	8.34E-06	3.86E-06		4.50E-06	4.54E-06	5.05E-06	3.94E-06		1.99E-05	4.25E-06	6.05E-06	7.89E-06	7.15E-06	5.07E-06	4.15E-06	4.03E-0
	TGI	1.99E-05	7.70E-06		8.85E-06	8.25E-06	9.24E-06	6.93E-06		> 1.99E-05	8.21E-06	1.47E-05	1.99E-05	> 1.99E-05	9.44E-06	7.29E-06	7.25E-0
19-AUG-02	LC50	> 1.99E-05	1.54E-05		1.74E-05	1.49E-05	1.70E-05	1.22E-05		> 1.99E-05	1.59E-05	> 1.99E-05	> 1.99E-05	> 1.99E-05	1.75E-05	1.28E-05	1.30E-0

Secondary Evaluation (Mol/L)											
Secondary Screening		% Macromolecules Synthesis			Apoptosis	DNA Binding	% Cytoskeleton				
		PROTEIN	DNA	RNA	NUCLEOSOMES	DIEM	ACTIN	TUBULIN	TELOMERASE		
PM - Fernando de la Calle020 20-AUG-02	IC50	-	-	1.57E-05	> 1.99E-05	-	-	-			

Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6mm diameter): 9.5 mm inhibition zone

Spectroscopic data

ESMS: *m/z* 511 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz): 6.51 (s, C-15), 4.04 (s, OMe), 3.75 (s, OMe), 2.23 (s, Me), 1.89 (s, Me), 0.84 (d, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): 186.32, 181.28, 175.83, 156.43, 145.27, 142.75, 141.05, 137.00, 132.63, 128.67, 126.64, 122.00, 120.69, 60.69, 60.21, 59.12, 58.04, 57.89, 50.12, 49.20, 46.72, 39.88, 32.22, 25.33, 21.29, 15.44, 8.23.

Example 5. Cross-feeding experiments

Heterologous expression of safracin biosynthetic precursors genes for P2 and P14 production

In the attempt to shed light on the mechanism of the P2 and P14 biosynthesis we have cloned and expressed the downstream NRPS genes to determine their biochemical activity.

To overproduce P14, *sacEFGH* genes were cloned (pB7983) (**Fig. 4**). To overproduce P2 in a heterologous system, *sacD* to *sacH* genes were cloned (pB5H83)(**Fig. 4**). For this purpose we PCR amplified fragments harboring the genes of interest using oligonucleotides that contain a *Xba*I restriction site at the 5' end. Oligonucleotides PFSC79 (5'-CGTCTAGACACCGGCTTCATGG-3') and PFSC83 (5'-GGTCTAGATAACAGCCAACAAACATA-3') were used to amplify *sacE* to *sacH* genes; and oligonucleotides 5HPT1-XB (5'-CATCTAGACCGGACTGATATTCG-3') and PFSC83 (5'-GGTCTAGATAACAGCCAACAAACATA-3') were used to amplify *sacD* to *sacH* genes. The PCR fragments digested with *Xba*I were cloned into the *Xba*I restriction site of the pBBR1-MCS2 plasmid (Kovach et al, *Gene* 1994, 166, 175-176). The two plasmids, pB7983 and pB5H83, were introduce separately into three heterologous bacteria *P. fluorescens* (CECT 378), *P. putida* (ATCC12633) and *P. stutzeri* (ATCC 17588) by conjugation (see table II). When culture broth of the fermentation of the transconjugant strains was checked by HPLC analysis, big amounts of P14 compound was visualized in the three strains containing pB7983 plasmid, whereas big amounts of P2 and some P14 product were observed when pB5H83 plasmid was expressed in the heterologa bacteria.

Cross-feeding

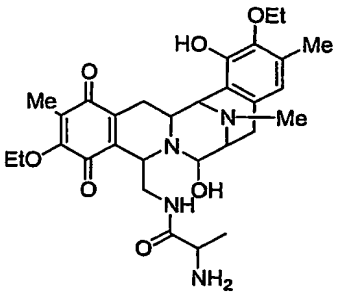
As it was shown in Example 4, the *sacF*- (PM-S1-008) and *sacG*- (PM-S1-009) mutants were not able to produce neither safracins nor P2 and P14 compounds. The addition of chemically synthesized P2 to these mutants during their fermentation yields safracin production.

Moreover, the co-cultivation of an heterologous strain of *P. stutzeri* (ATCC 17588) harboring plasmid pB5H83 (PM-18-004), which expression produces P2 and P14, with either one of the two mutants *sacF*- and *sacG*- resulted in safracin production. The co-cultivation of an heterologous strain *P. stutzeri* (ATCC 17588) harboring plasmid pB7983 (PM-18-005), which expression produces only P14, with either one of the two *P. fluorescens* A2-2 mutants mentioned before resulted in no safracin production at all. These results suggest that P14 is transformed into P2, a molecule that can easily be transported in and out through the *Pseudomonas* sp. cell wall and which presence it is absolutely necessary for the biosynthesis of safracin.

Example 6. Biological production of new "unnatural" molecules

The addition of 2g/L of an specific modified P2 derivative precursor, P3, a 3-hydroxy-5-methyl-*O*-methyltyrosine, to the *sacF* mutant (PM-S1-008) fermentation yielded two "unnatural" safracins that incorporated the modified precursor P3 in its structure, Safracin A(OEt) and Safracin B(OEt).

SAFRACIN B-Etoxi (Safracin B (OEt))



$C_{30}H_{40}N_4O_7$
Exact Mass: 568,29
Mol. Wt.: 568,66
C, 63,36; H, 7,09; N, 9,85; O, 19,69

Strain

saf F $^-$ mutant from *P.fluorescens* A2-2 (PM-S1-008)

Fermentation conditions:

Seed medium containing 1% glucose; 0.25% beef extract; 0.5% bacto-peptone; 0.25% NaCl; 0.8% CaCO3 was inoculated with 0.1% of a frozen vegetative stock of the microorganism, and incubated on a rotary shaker (250 rpm) at 27°C. After 30h of incubation, the 2% (v/v) seed culture of the mutant PM-S1-008 was transferred into 2000 ml Erlenmeyer flasks containing 250 ml of the M-16 B production medium, composed of 15.2 % mannitol; 3.5 % Dried brewer's yeast; 1.4 % $(NH_4)_2$ 0.001%; $FeCl_3$; 2.6 % CO_3Ca and 0.2% P3 (3-hydroxy-5-methyl-O-methyltyrosine) The temperature of the incubation was 27°C from the inoculation till 40 hours and then, 24°C to final process (71 hours). The pH was not controlled. The agitation of the rotatory shaker was 220 rpm with 5 cm eccentricity.

Isolation

4 x 2000/250 ml Erlenmeyer flasks were joined together (970 ml), centrifuged (12.000 rpm, 4°C, 10', J2-21 Centrifuge BECKMAN) to remove

cells. The clarified broth (765 ml) was adjusted to pH 9.0 by NaOH 10%. Then, the alkali-clarified broth was extracted with 1:1 (v/v) EtOAc (x2). The organic phase was evaporated under high vacuum and a greasy-dark extract was obtained (302 mg).

This extract was washed by an hexane trituration for removing impurities and the solids were purified by a chromatography column using Silica normal-phase and a mixture of Ethyl Acetate: Methanol (from 12:1 to 1:1). The fractions were analyzed under UV on TLC (Silica 60, mobile phase EtOAc:MeOH 5:4. Rf 0.3 (Safracin B-OEt and 0.15 Safracin A-OEt). From this, safracins B OEt (25 mg) and safracin A OEt (20 mg) were obtained.

Biological activities of safracin B (OEt)

Antitumor activities

Cells Lines (Mol/L)

Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix	
		DU145	LNCaP	SK-OV-3	IGROV	OVCAR-3	SK-BR-3	SK-MEL-28	SN12C	A549	K-562	PANC-1	HT-29	HCT-15	LOVO-DOX	HELA	HEp-2
Safracin B Etoxi 23-OCT-02	GI50	4.01E-07	4.84E-08		4.06E-08	6.82E-07	4.82E-08	1.69E-07		5.01E-07	3.97E-08	6.49E-07	2.44E-07	4.43E-07	2.09E-06	8.92E-08	7.70E-08
	TGI	1.01E-06	> 1.76E-05		9.97E-08	1.19E-06	1.16E-07	4.40E-07		1.16E-06	1.08E-07	2.06E-06	1.39E-06	1.09E-06	9.88E-06	3.16E-07	2.74E-07
	LC50	1.60E-05	8.28E-07		4.27E-06	6.37E-06	1.02E-06	1.13E-06		6.66E-06	3.69E-06	1.26E-05	> 1.76E-05	> 1.76E-05	> 1.76E-05	1.35E-06	9.76E-07

Secondary Evaluation (Mol/L)

Secondary Screening			Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton		
			PROTEIN	DNA	RNA	NUCLEOSOMES	DEL	ACTIN	TUBULIN	TELOMERASE
	10-OCT-02	IC50	> 1.76E-05	1.76E-06	1.76E-07	6.28E-08	1.76E-06	.	.	

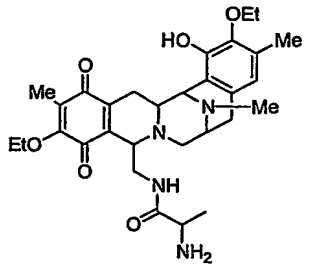
Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6 mm diameter): 17,5 mm inhibition zone

Spectroscopic data:

ESMS: m/z 551 $[M-H_2O+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz): 6.48 (s, H-15), 2.31 (s, 16-Me), 2.22 (s, 12-NMe), 1.88 (s, 6-Me), 1.43 (t, J = 6.9 Hz, Me-Etoxy), 1.35 (t, J = 6.9 Hz, Me-Etoxy), 0.81 (d, J = 7.2 Hz, H-26)

SAFRACIN A-Etoxi (Safracin A (OEt))



$C_{30}H_{40}N_4O_6$
Exact Mass: 552,29
Mol. Wt.: 552,66
C, 65,20; H, 7,30; N, 10,14; O, 17,37

Strain:

The same as for Safracin B (OEt)

Fermentation conditions:

The same as for Safracin B (OEt)

Isolation:

4 x 2000/250 ml Erlenmeyer flasks were joined together (970 ml), centrifuged (12.000 rpm, 4°C, 10', J2-21 Centrifuge BECKMAN) to remove cells. The clarified broth (765 ml) was adjusted to pH 9,0 by NaOH 10%. Then, the alkali-clarified broth was extracted with 1:1 (v/v) EtOAc (x2). The organic phase was evaporated under high vacuum and a greasy-dark extract was obtained (302 mg).

This extract was washed by an hexane trituration for removing impurities and the solids were purified by a chromatography column using Silica normal-phase and a mixture of Ethyl Acetate: Methanol (from 12:1 to 1:1). The fractions were analysed under UV on TLC (Silica 60, mobile phase

EtOAc:MeOH 5:4. Rf 0.3 Safracin B-OEt and 0.15 Safracin A-OEt). From this, safracins B OEt (25 mg) and safracin A OEt (20 mg) were obtained.

Biological activities of safracin A (OEt):

Antitumor activities

Cells Lines (Mol/L)																		
Primary Screening		Prostate		Ovary			Breast	Melanoma	Endothelio	NSCL	Leukemia	Pancreas	Colon			Cervix		
		LOU-1A	LNCaP	SK-OV-3	IGROV	IGROV-ET	SKBR3	SKMEL28	ETHUEDT	A549	K-562	CFPAC-1	HT29	LOVO	LOVO-DOX	HELA	HEACRT	
Safracin A Etoxi (OEt) . .	GI50	2.64E-06	3.78E-07		4.52E-07	2.01E-06	6.55E-07	7.98E-07		4.00E-06	3.11E-07	3.05E-06	1.97E-06	2.03E-06	6.72E-06	1.02E-06	7.64E-07	
	TGI	5.39E-06	7.42E-07		9.28E-07	5.10E-06	1.16E-06	1.90E-06		7.17E-06	6.86E-07	5.83E-06	4.41E-06	4.41E-06	9.84E-06	2.81E-06	2.32E-06	
23-OCT-02	LC50	1.10E-05	1.45E-06		1.76E-06	1.30E-05	5.57E-06	5.77E-06		1.28E-05	1.51E-06	1.11E-05	9.88E-06	9.61E-06	1.59E-05	7.85E-06	6.59E-06	

Secondary Evaluation (Mol/L)											
Secondary Screening		Macromolecules Synthesis			Apoptosis	DNA Binding	Cytoskeleton				
		PROTEIN	DNA	RNA	NUCLEOSOMES	GEL	ACTIN	TUBULIN	TELOMERASE		
10-OCT-02	LC50	.	.	6.33E-06	1.81E-06	.	.	.			

Antimicrobial activity: On solid medium

Bacillus subtilis. 10µg/disk (6 mm diameter): 10 mm inhibition zone

Spectroscopic data:

ESMS: m/z 553 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz): 6.48 (s, H-15), 2.33 (s, 16-Me), 2.21 (s, 12-NMe), 1.88 (s, 6-Me), 1.42 (t, J = 6.9 Hz, Me-Etoxy), 1.34 (t, J = 6.9 Hz, Me-Etoxy), 0.8 (d, J = 6.9 Hz, H-26)

Example 7. Enzymatic transformation of Safracin B into Safracin A

In order to assay the enzymatic activity of conversion of safracin B into safracin A, a 120 hours fermentation cultures (see conditions in Example.2.*Biological assay (biotest) for safracin production*) of different strains were collected and centrifuged (9.000 rpm x 20 min.). The strains assayed were *P. fluorescens* A2-2, as wild type strain, and *P.fluorescens* CECT378 + pBHPT3 (PM-19-006), as heterologous expression host. Supernatant were discarded and cells were washed (NaCl 0.9 %) twice and resuspended in 60 ml phosphate buffer 100 mM pH 7.2. 20 ml from the cell suspension was distributed into three Erlenmeyer flask:

A. Cell suspension + Safracin B (400 mg/L)
B. Cell suspension heated at 100 °C during 10 min. + Safracin B (400 mg/L) (negative control)
C. Cell suspension without Safracin B (negative control)

The biochemical reaction was incubated at 27 °C at 220 rpm and samples were taken every 10 min. Transformation of safracin B into safracin A was followed by HPLC. The results clearly demonstrated that the gene cloned in pBHPT3, *sacH*, codes for a protein responsible for the transformation of safracin B into safracin A.

Based on this results we did an assay to find out if this same enzyme was able to recognize a different substrate such as ecteinascidin 743 (ET-743) and transform this compound into Et-745 (with the C-21 hydroxy missing). The experiment above was repeated to obtain Erlenmeyer flasks containing:

A. Cell suspension + ET-743 (567 mg/L aprox.)

B. Cell suspension heated at 100 °C during 10 min. + ET-743(567 mg/L) (negative control)

C. Cell suspension without ET-743 (negative control)

The biochemical reaction was incubated at 27 °C at 220 rpm and samples were taken at o, 10 min, 1h, 2h, 3h, 4h, 20h, 40h, 44h, 48h. Transformation of ET-743 into ET-745 was followed by HPLC. The results clearly demonstrated that the gene cloned in pBHPT3, *sacH*, codes for a protein responsible for the transformation of Et-743 into Et-745. This demonstrates that this enzymes recognizes ecteinascidin as substrate and that it can be used in the biotransformation of a broad range of structures.

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Ser Thr Asp Ala Leu Leu Ala Pro Ser Arg Glu His Ile Gly Val Glu
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Arg Tyr Leu Val Phe Arg Ile His His Ile Ile Ala Asp Leu Trp Ser
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Val Gly Leu Leu Ile Arg Asp Phe Ala Glu Asp Cys Met Asp Arg Ser
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Ser Ile Thr Leu Ala Ser Arg Pro Ile Ala Pro Leu Ile Asp Pro Glu
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Phe Trp Arg His Gln Met Ser Gln Asp Thr Pro Phe Ser Leu Pro Met
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Val Ile Asp Gln Glu Ser Ser Ala Asp Leu Ala Arg Leu Ala Thr Ala
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725 730 735

Gly Ile Gly Val Pro Gly Glu Leu Ile Ile His Gly His Gly Val Ala
740 745 750

Gln Gly Tyr Val Ser Asp Pro Val Arg Ser Ala Ala Ser Phe Leu Pro
755 760 765

Ala Ser Asp Gly Leu Arg Cys Tyr Arg Thr Gly Asp Arg Val Arg Trp
770 775 780

Leu Pro Asp Gly Arg Leu Asp Phe Ile Gly Arg Glu Asp Asp Gln Val
785 790 795 800

Lys Val Arg Gly Phe Arg Val Glu Leu Gly Pro Val Gln Ala Ala Leu
805 810 815

His Ala Ile Glu Thr Ile His Glu Ser Ala Val Val Val Val Pro Lys
820 825 830

Gly Gln Gln Arg Ser Ile Val Ala Phe Ile Val Leu Lys Ala Pro Ser
835 840 845

Glu Asp Glu Ala Val Gln Arg Asn Asn Ile Lys Gln His Leu Leu Gly
850 855 860

Val Leu Pro Tyr Tyr Ala Leu Pro Asp Lys Phe Ile Phe Val Lys Ala

865 870 875 880

Leu Pro Arg Asn Thr His Gly Lys Ile Asp Arg Thr Leu Leu Leu Gln
885 890 895

His Glu Pro Gln Thr Glu Gln Glu Ser Ala Met Arg Asp Ala Thr Asp
900 905 910

Val Glu His Arg Ile Ala Asn Cys Trp Gln Thr Ile Ile Gly His Pro
915 920 925

Val Gln Leu His Glu Asn Phe Leu Asp Ile Gly Gly His Ser Leu Ser
930 935 940

Leu Thr His Leu Thr Gly Leu Leu Arg Lys Glu Phe Asn Ile His Ile
945 950 955 960

Ser Leu His Asp Leu Trp Ile Arg Pro Thr Ile Glu Gln Gln Ala Asp
965 970 975

Phe Ile His Lys Leu Gln Asn Ser Val Leu Thr Lys Pro Ala Ala Ala
980 985 990

Pro Ile Pro Arg Leu Asp Arg Lys Ile Ser His His
995 1000

<210> SEQ ID 3
<211> Lenght: 1062
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 3

Met Ser Val Asp Thr Cys Arg Thr Ala Thr Phe Pro Ala Ser Tyr Gly
1 5 10 15

Gln Glu Gln Ile Trp Phe Leu Asn Glu Leu Asn Pro His Ser Gln Leu
20 25 30

Ala Tyr Thr Leu Ala Met Lys Val Ser Ile Ala Gly Lys Leu Asn Thr
35 40 45

Leu Arg Leu Gln Arg Ala Val Asn Gln Val Val Ala Ser Gln Glu Ile
50 55 60

Leu Arg Thr Ser Phe Ala Tyr Lys Asn Gln Lys Leu Ser Gln Val Ile
65 70 75 80

Ser Pro Ser Ala Thr Leu Pro Ile Arg Ser Ala His Cys Ile Asp Asp
85 90 95

Val Pro Gly Leu Gln Arg Leu Ile Asn Met Glu Ala Gln Arg Gly Trp
100 105 110

Ser Leu Ser Ser Ala Pro Leu Tyr Arg Leu Leu Leu Ile Lys Thr Gly
115 120 125

Asp Gln Gln His Glu Leu Val Ile Cys Thr His His Ile Val Cys Asp
130 135 140

Gly Ile Ser Leu Gln Leu Leu Leu Gln Lys Ile Val Ser Ala Tyr Gln
145 150 155 160

Gly Gln Ser Asp Gly Arg Val Leu Thr Ser Pro Asp Glu Glu Thr Leu
165 170 175

Gln Phe Val Asp Tyr Ala Ala Trp Ser Arg Gln His Glu Tyr Ala Gly
180 185 190

Leu Glu Tyr Trp Arg Gln Gln Leu Ala Asp Ala Pro Thr Ile Leu Asp
195 200 205

Ile Ser Thr Lys Thr Gly Arg Ser Glu Gln Gln Thr Phe Leu Gly Ala
210 215 220

Arg Ile Pro Val Glu Phe Ser His His Gln Trp Gln Ala Leu Arg Gln
225 230 235 240

Ile Phe Arg Pro Gln Gly Ile Ser Cys Ala Ala Val Phe Leu Ala Ala
245 250 255

Tyr Cys Val Val Leu His Arg Leu Ala Glu Gln Asp Asp Ile Leu Ile
260 265 270

Gly Leu Pro Thr Ser Asn Arg Leu Arg Pro Glu Leu Ala Gln Val Ile
275 280 285

Gly Tyr Leu Ser Asn Leu Cys Val Phe Arg Ser Gln Tyr Ala His Asp
290 295 300

Gln Ser Val Thr Asp Phe Leu Gln Gln Val Gln Leu Thr Leu Pro Asn
305 310 315 320

Leu Ile Glu His Gly Glu Thr Pro Phe Gln Gln Val Leu Glu Ser Val
325 330 335

Glu His Thr Arg Gln Ala Gly Val Thr Pro Leu Cys Gln Val Leu Phe
340 345 350

Gly Tyr Glu Gln Asp Val Arg Arg Thr Leu Asp Ile Gly Asp Leu Gln
355 360 365

Leu Thr Val Ser Asp Val Asp Thr Gly Ala Ala Arg Leu Asp Leu Ser
370 375 380

Leu Phe Leu Phe Glu Asp Glu Leu Asn Val Cys Gly Phe Leu Glu Tyr
385 390 395 400

Ala Thr Asp Arg Ile Asp Ala Ala Ser Ala Gln Asn Met Val Arg Met
405 410 415

Leu Ser Ser Val Leu Arg Glu Phe Val Ala Ala Pro Gln Ala Pro Leu
420 425 430

Ser Glu Val Gln Leu Gly Ala Ala Asp Ser Gln Ala Gln Thr Pro Ala
435 440 445

Ile Ala Pro Ala Phe Pro Ser Val Pro Ala Arg Leu Phe Ala Leu Ala
450 455 460

Asp Ser His Pro Asn Ala Thr Ala Leu Arg Asp Glu Gln Gly Glu Leu
465 470 475 480

Thr Tyr Ala Gln Val Cys Gln Gln Ile Leu Gln Ala Ala Ala Thr Leu
485 490 495

Arg Ala Gln Gly Ala Lys Pro Gly Thr Leu Ile Ala Val Ile Gly Glu
500 505 510

Arg Gly Asn Pro Trp Leu Ile Ala Met Leu Ala Ile Trp Gln Val Gly

515 520 525

Gly Ile Tyr Val Pro Leu Ser Lys Asp Leu Pro Glu Gln Arg Leu Gln
530 535 540

Gly Ile Leu Ala Glu Leu Glu Gly Ala Ile Leu Ile Thr Asp Asp Thr
545 550 555 560

Thr Pro Glu Arg Phe Arg Gln Arg Val Thr Leu Pro Met His Ala Leu
565 570 575

Trp Ala Asp Gly Ala Thr His His Glu Arg Gln Thr Thr Asp Ala Ser
580 585 590

Arg Leu Ser Gly Tyr Met Met Tyr Thr Ser Gly Ser Thr Gly Lys Pro
595 600 605

Lys Gly Val His Val Ser Gln Ala Asn Leu Val Ala Thr Leu Ser Ala
610 615 620

Phe Gly Gln Leu Leu Gln Val Lys Pro Ser Asp Arg Met Leu Ala Leu
625 630 635 640

Thr Thr Phe Ser Phe Asp Ile Ser Leu Leu Glu Leu Leu Leu Pro Leu
645 650 655

Val Gln Gly Ala Ser Val Gln Ile Ala Val Ala Gln Ala Gln Arg Asp
660 665 670

Ala Glu Lys Leu Ala Gly Tyr Leu Ala Asp Pro Arg Ile Thr Leu Val
675 680 685

Gln Ala Thr Pro Val Thr Trp Arg Leu Leu Leu Ser Thr Gly Trp Gln
690 695 700

Pro Arg Glu Ser Leu Thr Leu Leu Cys Gly Gly Glu Ala Leu Pro Gln
705 710 715 720

Asp Leu Ala Asp Arg Leu Cys Leu Pro Gly Met Thr Leu Trp Asn Leu
725 730 735

Tyr Gly Pro Thr Glu Thr Thr Ile Trp Ser Thr Ala Cys Arg Leu Gln
740 745 750

Pro Gly Ala Pro Val Gln Leu Gly His Pro Ile Ala Gly Thr Gln Ile
755 760 765

Ala Leu Val Asp Arg Asn Leu Arg Ser Val Pro Arg Gly Val Ile Gly
770 775 780

Glu Leu Leu Ile Cys Gly Pro Gly Val Ser Gln Gly Tyr Tyr Arg Asn
785 790 795 800

Pro Val Glu Thr Ala Lys Arg Phe Val Pro Asp Pro His Gly Ser Gly
805 810 815

Lys Arg Ala Tyr Leu Thr Gly Asp Arg Met Arg Met Gln Gln Asp Gly
820 825 830

Ser Leu Ala Tyr Ile Gly Arg Arg Asp Asp Gln Ile Lys Leu Arg Gly
835 840 845

His Arg Ile Glu Leu Gly Glu Ile Glu Thr Ala Leu Arg Lys Leu Pro
850 855 860

Gly Val Arg Asp Ala Ala Ala Gln Leu His Asp Gln Asp Pro Ser Arg
865 870 875 880

Gly Ile Gln Ala Phe Val Gln Leu Cys Ala Thr Val Asp Glu Ser Leu
885 890 895

Ile Asp Ile Gly Gln Trp Leu Glu Thr Leu Arg Gln Thr Leu Pro Glu
900 905 910

Ala Trp Leu Pro Thr Glu Tyr Tyr Arg Ile Asp Gly Ile Pro Leu Thr
915 920 925

Tyr Asn Gly Lys Arg Asp Arg Lys Arg Leu Leu His Gln Ala Val Arg
930 935 940

Leu Gln Thr Leu Ser Leu Arg Val Ala Pro Ser Ser Asp Thr Glu Thr
945 950 955 960

Arg Val Gln Gln Ile Trp Cys Glu Leu Leu Gly Leu Glu Asp Ile Gly
965 970 975

Val Thr Asp Asp Phe Phe Gln Leu Gly Gly His Ser Ile Leu Val Ala
980 985 990

Arg Met Val Glu Arg Ile Glu Thr Ala Phe Gly Arg Arg Val Pro Ile
995 1000 1005

Ala Asp Ile Tyr Phe Ser Pro Thr Ile Ala Arg Val Ala Ala Thr
1010 1015 1020

Leu Asp Ser Met Thr Phe Glu Gln Gly Leu Ala Ala His Ser Val
1025 1030 1035

Lys Gly Asp Trp Glu Phe Thr Ala Ile Ser Leu Gln His Asn Ala
1040 1045 1050

Asp Ser Thr Ala Ala Ala Gln Glu Arg
1055 1060

<210> SEQ ID 4
<211> Length: 1432
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 4

Met His Ser Pro Thr Ile Asp Thr Phe Glu Ala Ala Leu Arg Ser Leu
1 5 10 15

Pro Ala Ala Arg Asp Ala Leu Gly Ala Tyr Pro Leu Ser Ser Glu Gln
20 25 30

Lys Arg Leu Trp Leu Leu Ala Gln Leu Ala Gly Thr Ala Thr Leu Pro
35 40 45

Val Thr Val Arg Tyr Ala Phe Thr Gly Thr Val Asp Leu Ala Val Val
50 55 60

Gln Gln Asn Leu Ser Ala Trp Ile Ala His Ser Glu Ser Leu Arg Ser
65 70 75 80

Leu Phe Val Glu Val Leu Glu Arg Pro Val Arg Leu Leu Met Pro Thr

85 90 95

Gly Leu Val Lys Leu Glu Tyr Phe Asp Arg Pro Pro Ser Asp Ala Asp
100 105 110

Met Ala Glu Leu Ile Gly Ala Ala Phe Glu Leu Asp Lys Gly Pro Leu
115 120 125

Leu Arg Ala Phe Ile Thr Arg Thr Ala Ala Gln Gln His Glu Leu His
130 135 140

Leu Val Gly His Pro Ile Val Val Asp Glu Pro Ser Leu Gln Arg Ile
145 150 155 160

Ala Gln Thr Leu Phe Gln Thr Glu Pro Asp His Gln Tyr Pro Ala Val
165 170 175

Gly Ala Ile Ala Glu Val Phe Gln Arg Glu Gln Thr Leu Ala Gln Asp
180 185 190

Ala Gln Ile Thr Glu Gln Trp Gln Gln Trp Gly Ile Gly Leu Gln Ala
195 200 205

Pro Ala Ala Thr Glu Ile Pro Thr Glu Asn Pro Arg Pro Ala Ile Lys
210 215 220

Gly Ser Asp Arg Gln Val His Glu Ala Leu Thr Ala Trp Gly Asp Gln
225 230 235 240

Pro Val Ala Glu Ala Glu Ile Val Ser Ser Trp Leu Thr Val Leu Met
245 250 255

Arg Trp Gln Gly Ser Gln Ser Ala Leu Cys Ala Ile Lys Val Arg Asp
260 265 270

Lys Ala His Ala Asn Leu Ile Gly Pro Leu Gln Thr Tyr Leu Pro Val
275 280 285

Arg Val Asp Met Pro Asp Gly Ser Thr Leu Ala Gln Leu Arg Leu Gln
290 295 300

Val Glu Glu Gln Leu Asn Gly Asn Asp His Pro Ser Phe Ser Thr Leu
305 310 315 320

Leu Glu Val Cys Pro Pro Lys Arg Asp Leu Ser Arg Thr Pro Tyr Phe
325 330 335

Gln Thr Gly Leu Gln Phe Ile Ala His Asp Val Glu Gln Arg Asp Phe
340 345 350

His Ala Gly Asn Leu Thr Arg Leu Pro Thr Lys Gln Pro Ser Ser Asp
355 360 365

Leu Asp Leu Phe Ile Ser Cys Trp Val Ser Asp Gly Thr Leu Gly Leu
370 375 380

Thr Leu Asp Tyr Asp Cys Ala Val Leu Asn Ser Ser Gln Val Glu Val
385 390 395 400

Leu Ala Gln Ala Leu Ile Ser Val Leu Ser Ala Pro Gly Glu Gln Pro
405 410 415

Ile Ala Thr Val Ala Leu Met Gly Gln Gln Met Gln Gln Thr Val Leu
420 425 430

Ala Gln Ala His Gly Pro Arg Thr Thr Pro Pro Gln Leu Thr Leu Thr
435 440 445

Glu Trp Val Ala Ala Ser Thr Glu Lys Ser Pro Leu Ala Val Ala Val
450 455 460

Ile Asp His Gly Gln Gln Leu Ser Tyr Ala Glu Leu Trp Ala Arg Ala
465 470 475 480

Ala Leu Val Ala Ala Asn Ile Ser Gln His Val Ala Lys Pro Arg Ser
485 490 495

Ile Ile Ala Val Ala Leu Pro Arg Ser Ala Glu Phe Ile Ala Ala Leu
500 505 510

Leu Gly Val Val Arg Ala Gly His Ala Phe Leu Pro Ile Asp Pro Arg
515 520 525

Leu Pro Thr Asp Arg Ile Gln Phe Leu Ile Glu Asn Ser Gly Cys Glu
530 535 540

Leu Val Ile Thr Ser Asp Gln Gln Ser Val Glu Gly Trp Pro Gln Val
545 550 555 560

Ala Arg Ile Arg Met Glu Ala Leu Asp Pro Asp Ile Arg Trp Val Ala
565 570 575

Pro Thr Gly Leu Ser His Ser Asp Ala Ala Tyr Leu Ile Tyr Thr Ser
580 585 590

Gly Ser Thr Gly Val Pro Lys Gly Val Val Val Glu His Arg Gln Val
595 600 605

Val Asn Asn Ile Leu Trp Arg Gln Arg Thr Trp Pro Leu Thr Ala Gln
610 615 620

Asp Asn Val Leu His Asn His Ser Phe Ser Phe Asp Pro Ser Val Trp
625 630 635 640

Ala Leu Phe Trp Pro Leu Leu Thr Gly Gly Thr Ile Val Leu Ala Asp
645 650 655

Val Arg Thr Met Glu Asp Ser Thr Ala Leu Leu Asp Leu Met Ile Arg
660 665 670

His Asp Val Ser Val Leu Gly Gly Val Pro Ser Leu Leu Gly Thr Leu
675 680 685

Ile Asp His Pro Phe Ala Asn Asp Cys Arg Ala Val Lys Leu Val Leu
690 695 700

Ser Gly Gly Glu Val Leu Asn Pro Glu Leu Ala His Lys Ile Gln Lys
705 710 715 720

Val Trp Gln Ala Asp Val Ala Asn Leu Tyr Gly Pro Thr Glu Ala Thr
725 730 735

Ile Asp Ala Leu Tyr Phe Ser Ile Asp Lys Asn Ala Ala Gly Ala Ile
740 745 750

Pro Ile Gly Tyr Pro Ile Asp Asn Thr Asp Ala Tyr Ile Val Asp Leu
755 760 765

Asn Leu Asn Pro Val Pro Pro Gly Val Pro Gly Glu Ile Met Leu Ala
770 775 780

Gly Gln Asn Leu Ala Arg Gly Tyr Leu Gly Lys Pro Ala Gln Thr Ala
785 790 795 800

Gln Arg Phe Leu Pro Asn Pro Phe Gly Asn Gly Arg Val Tyr Ala Thr
805 810 815

Gly Asp Leu Gly Arg Arg Trp Ser Ser Gly Ala Ile Ser Tyr Leu Gly
820 825 830

Arg Arg Asp Gln Gln Val Lys Ile Arg Gly His Arg Ile Glu Leu Asn
835 840 845

Glu Val Ala His Leu Leu Cys Gln Ala Leu Glu Leu Lys Glu Ala Ile
850 855 860

Val Phe Ala Gln His Ala Gly Thr Glu Gln Ala Arg Leu Val Ala Ala
865 870 875 880

Ile Glu Gln Gln Pro Gly Leu His Ser Glu Gly Ile Lys Gln Glu Leu
885 890 895

Leu Arg His Leu Pro Ala Tyr Leu Ile Pro Ser Gln Leu Leu Leu Leu
900 905 910

Asp Glu Leu Pro Arg Thr Ala Thr Gly Lys Val Asp Met Leu Lys Leu
915 920 925

Asp Gln Leu Ala Ala Pro Gln Leu Asn Asp Ala Gly Gly Thr Glu Cys
930 935 940

Arg Ala Pro Arg Thr Asp Leu Glu Gln Ser Val Met Thr Asp Phe Ala
945 950 955 960

Gln Val Leu Gly Leu Thr Ala Val Thr Pro Asp Thr Asp Phe Phe Glu
965 970 975

Gln Gly Gly Asn Ser Ile Leu Leu Thr Arg Leu Ala Gly Thr Leu Ser
980 985 990

Ala Lys Tyr Gln Val Gln Ile Pro Leu His Glu Phe Phe Leu Thr Pro

995 1000 1005

Thr Pro Ala Ala Val Ala Gln Ala Ile Glu Ile Tyr Arg Arg Glu
1010 1015 1020

Gly Leu Thr Ala Leu Leu Ser Arg Gln His Ala Gln Thr Leu Glu
1025 1030 1035

Gln Asp Ile Tyr Leu Glu Glu His Ile Arg Pro Asp Gly Leu Pro
1040 1045 1050

His Ala Asn Trp Tyr Gln Pro Ser Val Val Phe Leu Thr Gly Ala
1055 1060 1065

Thr Gly Tyr Leu Gly Leu Tyr Leu Ile Glu Gln Leu Leu Lys Arg
1070 1075 1080

Thr Thr Ser Arg Val Ile Cys Leu Cys Arg Ala Lys Asp Ala Glu
1085 1090 1095

His Ala Lys Ala Arg Ile Leu Glu Gly Leu Lys Thr Tyr Arg Ile
1100 1105 1110

Asp Val Gly Ser Glu Leu His Arg Val Glu Tyr Leu Thr Gly Asp
1115 1120 1125

Leu Ala Leu Pro His Leu Gly Leu Ser Glu His Gln Trp Gln Thr
1130 1135 1140

Leu Ala Glu Glu Val Asp Val Ile Tyr His Asn Gly Ala Leu Val
1145 1150 1155

Asn Phe Val Tyr Pro Tyr Ser Ala Leu Lys Ala Thr Asn Val Gly
1160 1165 1170

Gly Thr Gln Ala Ile Leu Glu Leu Ala Cys Thr Ala Arg Leu Lys
1175 1180 1185

Ser Val Gln Tyr Val Ser Thr Val Asp Thr Leu Leu Ala Thr His
1190 1195 1200

Val Pro Arg Pro Phe Ile Glu Asp Asp Ala Pro Leu Arg Ser Ala
1205 1210 1215

Val Gly Val Pro Val Gly Tyr Thr Gly Ser Lys Trp Val Ala Glu
1220 1225 1230

Gly Val Ala Asn Leu Gly Leu Arg Arg Gly Ile Pro Val Ser Ile
1235 1240 1245

Phe Arg Pro Gly Leu Ile Leu Gly His Thr Glu Thr Gly Ala Ser
1250 1255 1260

Gln Ser Ile Asp Tyr Leu Leu Val Ala Leu Arg Gly Phe Leu Pro
1265 1270 1275

Met Gly Ile Val Pro Asp Tyr Pro Arg Ile Phe Asp Ile Val Pro
1280 1285 1290

Val Asp Tyr Val Ala Ala Ala Ile Val His Ile Ser Met Gln Pro
1295 1300 1305

Gln Gly Arg Asp Lys Phe Phe His Leu Phe Asn Pro Ala Pro Val
1310 1315 1320

Thr Ile Arg Gln Phe Cys Asp Trp Ile Arg Glu Phe Gly Tyr Glu
1325 1330 1335

Phe Lys Leu Val Asp Phe Glu His Gly Arg Gln Gln Ala Leu Ser
1340 1345 1350

Val Pro Pro Gly His Leu Leu Tyr Pro Leu Val Pro Leu Ile Arg
1355 1360 1365

Asp Ala Asp Pro Leu Pro His Arg Ala Leu Asp Pro Asp Tyr Ile
1370 1375 1380

His Glu Val Asn Pro Ala Leu Glu Cys Lys Gln Thr Leu Glu Leu
1385 1390 1395

Leu Ala Ser Ser Asp Ile Thr Leu Ser Lys Thr Thr Lys Ala Tyr
1400 1405 1410

Ala His Thr Ile Leu Arg Tyr Leu Ile Asp Thr Gly Phe Met Ala
1415 1420 1425

Lys Pro Gly Val
1430

<210> SEQ ID 5
<211> Lenght: 350
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 5

Met Glu Ser Ile Ala Phe Pro Ile Ala His Lys Pro Phe Ile Leu Gly
1 5 10 15

Cys Pro Glu Asn Leu Pro Ala Thr Glu Arg Ala Leu Ala Pro Ser Ala
20 25 30

Ala Met Ala Arg Gln Val Leu Glu Tyr Leu Glu Ala Cys Pro Gln Ala
35 40 45

Lys Asn Leu Glu Gln Tyr Leu Gly Thr Leu Arg Glu Val Leu Ala His
50 55 60

Leu Pro Cys Ala Ser Thr Gly Leu Met Thr Asp Asp Pro Arg Glu Asn
65 70 75 80

Gln Glu Asn Arg Asp Asn Asp Phe Ala Phe Gly Ile Glu Arg His Gln
85 90 95

Gly Asp Thr Val Thr Leu Met Val Lys Ala Thr Leu Asp Ala Ala Ile
100 105 110

Gln Thr Gly Glu Leu Val Gln Arg Ser Gly Thr Ser Leu Asp His Ser
115 120 125

Glu Trp Ser Asp Met Met Ser Val Ala Gln Val Ile Leu Gln Thr Ile
130 135 140

Ala Asp Pro Arg Val Met Pro Glu Ser Arg Leu Thr Phe Gln Ala Pro
145 150 155 160

Lys Ser Lys Val Glu Glu Asp Asp Gln Asp Pro Leu Arg Arg Trp Val
165 170 175

```
Arg Gly His Leu Leu Phe Met Val Leu Cys Gln Gly Met Ser Leu Cys
            180                 185                 190

Thr Asn Leu Leu Ile Ser Ala Ala His Asp Lys Asp Leu Glu Leu Ala
        195                 200                 205

Cys Ala Gln Ala Asn Arg Leu Ile Gln Leu Met Asn Ile Ser Arg Ile
    210                 215                 220

Thr Leu Glu Phe Ala Thr Asp Leu Asn Ser Gln Gln Tyr Val Ser Gln
225                 230                 235                 240

Ile Arg Pro Thr Leu Met Pro Ala Ile Ala Pro Pro Lys Met Ser Gly
                245                 250                 255

Ile Asn Trp Arg Asp His Val Val Met Ile Arg Trp Met Arg Gln Ser
            260                 265                 270

Thr Asp Ala Trp Asn Phe Ile Glu Gln Ala Tyr Pro Gln Leu Ala Glu
        275                 280                 285

Arg Met Arg Thr Thr Leu Ala Gln Val Tyr Ser Ala His Arg Gly Val
    290                 295                 300

Cys Glu Lys Phe Val Gly Glu Glu Asn Thr Ser Leu Leu Ala Lys Glu
305                 310                 315                 320

Asn Ala Thr Asn Thr Ala Gly Gln Val Leu Glu Asn Leu Lys Lys Ser
                325                 330                 335

Arg Leu Lys Tyr Leu Lys Thr Lys Gly Cys Ala Gly Ala Gly
            340                 345                 350
```

<210> SEQ ID 6
<211> Lenght: 61
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 6

```
Met Pro Thr Phe Leu Gly Asp Asp Asp Ala Val Pro Cys Val Val Val
1               5                   10                  15
```

```
Val Asn Ala Asp Lys His Tyr Ser Ile Trp Pro Ser Ala Arg Asp Ile
            20                  25                  30

Pro Ser Gly Trp Ser Glu Glu Gly Phe Lys Gly Ser Arg Ser Asp Cys
        35                  40                  45

Leu Glu His Ile Ala Gln Ile Trp Pro Glu Pro Thr Ala
    50                  55                  60
```

<210> SEQ ID 7
<211> Lenght: 355
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 7

```
Met Thr Ser Thr His Arg Thr Thr Asp Gln Val Lys Pro Ala Val Leu
1               5                   10                  15

Asp Met Pro Gly Leu Ser Gly Ile Leu Phe Gly His Ala Ala Phe Gln
            20                  25                  30

Tyr Leu Arg Ala Ser Cys Glu Leu Asp Leu Phe Glu His Val Arg Asp
        35                  40                  45

Leu Arg Glu Ala Thr Lys Glu Ser Ile Ser Ser Arg Leu Lys Leu Gln
    50                  55                  60

Glu Arg Ala Ala Asp Ile Leu Leu Leu Gly Ala Thr Ser Leu Gly Met
65                  70                  75                  80

Leu Val Lys Glu Asn Gly Ile Tyr Arg Asn Ala Asp Val Val Glu Asp
                85                  90                  95

Leu Met Ala Thr Asp Asp Trp Gln Arg Phe Lys Asp Thr Val Ala Phe
            100                 105                 110

Glu Asn Tyr Ile Val Tyr Glu Gly Gln Leu Asp Phe Thr Glu Ser Leu
        115                 120                 125

Gln Lys Asn Thr Asn Val Gly Leu Gln Arg Phe Pro Gly Glu Gly Arg
    130                 135                 140
```

Asp Leu Tyr His Arg Leu His Gln Asn Pro Lys Leu Glu Asn Val Phe
145 150 155 160

Tyr Arg Tyr Met Arg Ser Trp Ser Glu Leu Ala Asn Gln Asp Leu Val
165 170 175

Lys His Leu Asp Leu Ser Arg Val Lys Lys Leu Leu Asp Ala Gly Gly
180 185 190

Gly Asp Ala Val Asn Ala Ile Ala Leu Ala Lys His Asn Glu Gln Leu
195 200 205

Asn Val Thr Val Leu Asp Ile Asp Asn Ser Ile Pro Val Thr Gln Gly
210 215 220

Lys Ile Asn Asp Ser Gly Leu Ser His Arg Val Lys Ala Gln Ala Leu
225 230 235 240

Asp Ile Leu His Gln Ser Phe Pro Glu Gly Tyr Asp Cys Ile Leu Phe
245 250 255

Ala His Gln Leu Val Ile Trp Thr Leu Glu Glu Asn Thr His Met Leu
260 265 270

Arg Lys Ala Tyr Asp Ala Leu Pro Glu Gly Gly Arg Val Val Ile Phe
275 280 285

Asn Ser Met Ser Asn Asp Glu Gly Asp Gly Pro Val Met Ala Ala Leu
290 295 300

Asp Ser Val Tyr Phe Ala Cys Leu Pro Ala Glu Gly Gly Met Ile Tyr
305 310 315 320

Ser Trp Lys Gln Tyr Glu Val Cys Leu Ala Glu Ala Gly Phe Lys Asn
325 330 335

Pro Val Arg Thr Ala Ile Pro Gly Trp Thr Pro His Gly Ile Ile Val
340 345 350

Ala Tyr Lys
355

<210> SEQ ID 8
<211> Lenght 347
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE 8

```
Met Ala Arg Ser Pro Glu Thr Asn Ser Ala Met Pro Gln Gln Ile Arg
1               5                  10                  15

Gln Leu Leu Tyr Ser Gln Leu Ile Ser Gln Ser Ile Gln Thr Phe Cys
            20                  25                  30

Glu Leu Arg Leu Pro Asp Val Leu Gln Ala Ala Gly Gln Pro Thr Ser
        35                  40                  45

Ile Glu Arg Leu Ala Glu Gln Thr His Thr His Ile Ser Ala Leu Ser
    50                  55                  60

Arg Leu Leu Lys Ala Leu Lys Pro Phe Gly Leu Val Lys Glu Thr Asp
65                  70                  75                  80

Glu Gly Phe Ser Leu Thr Asp Leu Gly Ala Ser Leu Thr His Asp Ala
                85                  90                  95

Phe Ala Ser Ala Gln Pro Ser Ala Leu Leu Ile Asn Gly Glu Met Gly
            100                 105                 110

Gln Ala Trp Arg Gly Met Ala Gln Thr Ile Arg Thr Gly Glu Ser Ser
        115                 120                 125

Phe Lys Met Tyr Tyr Gly Ile Ser Leu Phe Glu Tyr Phe Glu Gln His
    130                 135                 140

Pro Glu Arg Arg Ala Ile Phe Asp Arg Ser Gln Asp Met Gly Leu Asp
145                 150                 155                 160

Leu Glu Ile Pro Glu Ile Leu Glu Asn Ile Asn Leu Asn Asp Gly Glu
                165                 170                 175

Asn Ile Val Asp Val Gly Gly Gly Ser Gly His Leu Leu Met His Met
            180                 185                 190
```

Leu Asp Lys Trp Pro Glu Ser Thr Gly Ile Leu Phe Asp Leu Pro Val
195 200 205

Ala Ala Lys Ile Ala Gln Gln His Leu His Lys Ser Gly Lys Ala Gly
210 215 220

Cys Phe Glu Ile Val Ala Gly Asp Phe Phe Lys Ser Leu Pro Asp Ser
225 230 235 240

Gly Ser Val Tyr Leu Leu Ser His Val Leu His Asp Trp Gly Asp Glu
245 250 255

Asp Cys Lys Ala Ile Leu Ala Thr Cys Arg Arg Ser Met Pro Asp Asn
260 265 270

Ala Leu Leu Val Val Val Asp Leu Val Ile Asp Gln Ser Glu Ser Ala
275 280 285

Gln Pro Asn Pro Thr Gly Ala Met Met Asp Leu Tyr Met Leu Ser Leu
290 295 300

Phe Gly Ile Ala Gly Gly Lys Glu Arg Asn Glu Asp Glu Phe Arg Thr
305 310 315 320

Leu Ile Glu Asn Ser Gly Phe Asn Val Lys Gln Val Lys Arg Leu Pro
325 330 335

Ser Gly Asn Gly Ile Ile Phe Ala Tyr Pro Lys
340 345

<210> SEQ ID 9
<211> Lenght: 180
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 9

Met Ser Thr Leu Val Tyr Tyr Val Ala Ala Thr Leu Asp Gly Tyr Ile
1 5 10 15

Ala Thr Gln Gln His Lys Leu Asp Trp Leu Glu Asn Phe Ala Leu Gly
20 25 30

```
Asp Asp Ala Thr Ala Tyr Asp Asp Phe Tyr Gln Thr Ile Gly Ala Val
        35                  40                  45

Val Met Gly Ser Gln Thr Tyr Glu Trp Ile Met Ser Asn Ala Pro Asp
    50                  55                  60

Asp Trp Pro Tyr Gln Asp Val Pro Ala Phe Val Met Ser Asn Arg Asp
65                  70                  75                  80

Leu Ser Ala Pro Ala Asn Leu Asp Ile Thr Phe Leu Arg Gly Asp Ala
                85                  90                  95

Ser Ala Ile Ala Val Arg Ala Arg Gln Ala Ala Lys Gly Lys Asn Val
            100                 105                 110

Trp Leu Val Gly Gly Gly Lys Thr Ala Ala Cys Phe Ala Asn Ala Gly
        115                 120                 125

Glu Leu Gln Gln Leu Phe Ile Thr Thr Ile Pro Thr Phe Ile Gly Thr
    130                 135                 140

Gly Val Pro Val Leu Pro Val Asp Arg Ala Leu Glu Val Val Leu Arg
145                 150                 155                 160

Glu Gln Arg Thr Leu Gln Ser Gly Ala Met Glu Cys Ile Leu Asp Val
                165                 170                 175

Lys Lys Ala Asp
            180
```

<210> SEQ ID 10
<211> Length: 220
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 10

```
Met Ser Asn Val Phe Ser Gly Gly Lys Gly Asn Gly Asn Pro Gly Phe
1               5                   10                  15

Val Arg Thr Phe Ser Arg Ile Ala Pro Thr Tyr Glu Glu Lys Tyr Gly
            20                  25                  30

Thr Lys Leu Ser Gln Ala His Asp Asp Cys Leu Arg Met Leu Ser Arg
```

35 40 45

Trp Met Cys Thr Ser Arg Pro Glu Arg Val Leu Asp Ile Gly Cys Gly
50 55 60

Thr Gly Ala Leu Ile Glu Arg Met Phe Ala Leu Trp Pro Glu Ala Arg
65 70 75 80

Phe Glu Gly Val Asp Pro Ala Gln Gly Met Val Asp Glu Ala Ala Lys
85 90 95

Arg Arg Pro Phe Ala Ser Phe Val Lys Gly Val Ala Glu Ala Leu Pro
100 105 110

Phe Pro Ser Gln Ser Met Asp Leu Val Val Cys Ser Met Ser Phe Gly
115 120 125

His Trp Ala Asp Lys Ser Val Ser Leu Asn Glu Val Arg Arg Val Leu
130 135 140

Lys Pro Gln Gly Leu Phe Cys Leu Val Glu Asn Leu Pro Ala Gly Trp
145 150 155 160

Gly Leu Thr Thr Leu Ile Asn Trp Leu Leu Gly Ser Leu Ala Asp Tyr
165 170 175

Arg Ser Glu His Glu Val Ile Gln Leu Ala Gln Thr Ala Gly Leu Gln
180 185 190

Ser Met Glu Thr Ser Val Thr Asp Gln His Val Ile Val Ala Thr Phe
195 200 205

Arg Pro Cys Cys Gly Glu Val Gly Asp His Gly Arg
210 215 220

<210> SEQ ID 11
<211> Length: 509
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 11

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Gly Leu Ala Val Ala Ala Ala Leu Ala Glu Leu Gly Ile Ala Val Asp
20 25 30

Leu Ile Asp Lys Arg Pro Ala Ala Ser Pro His Ser Arg Ala Phe Gly
35 40 45

Leu Glu Pro Val Thr Leu Glu Leu Leu Asn Ala Trp Gly Val Ala Asp
50 55 60

Glu Met Ile Arg Arg Gly Ile Val Trp Ala Ser Ala Pro Leu Gly Asp
65 70 75 80

Lys Ala Gly Arg Thr Leu Ser Phe Ser Lys Leu Pro Cys Glu Tyr Pro
85 90 95

His Met Val Ile Ile Pro Gln Ser Gln Thr Glu Ser Val Leu Thr Asp
100 105 110

Trp Val Asn Arg Lys Gly Val Asn Leu Lys Arg Gly Tyr Ala Leu Lys
115 120 125

Ala Leu Asp Ala Gly Asp Leu His Val Glu Val Thr Leu Glu His Ser
130 135 140

Glu Thr Gly Ser Val Gln Gln Ser Arg Tyr Asp Trp Val Leu Gly Ala
145 150 155 160

Asp Gly Val Asn Ser Ser Val Arg Gln Leu Leu Asn Ile Ser Phe Val
165 170 175

Gly Gln Asp Tyr Lys His Ser Leu Val Val Ala Asp Val Val Leu Arg
180 185 190

Asn Pro Pro Ser Pro Ala Val His Ala Arg Ser Val Ser Arg Gly Leu
195 200 205

Val Ala Leu Phe Pro Leu Pro Asp Gly Ser Tyr Arg Val Ser Ile Glu
210 215 220

Asp Asn Glu Arg Met Asp Thr Pro Val Lys Gln Pro Val Thr His Glu
225 230 235 240

Glu Ile Ala Gly Gly Met Lys Asp Ile Leu Gly Thr Asp Phe Gly Leu
245 250 255

Ala Gln Val Leu Trp Ser Ala Arg Tyr Arg Ser Gln Gln Arg Leu Ala
260 265 270

Thr His Tyr Arg Gln Gly Arg Val Phe Leu Leu Gly Asp Ala Ala His
275 280 285

Thr His Val Pro Ala Gly Gly Gln Gly Leu Gln Met Gly Ile Gly Asp
290 295 300

Ala Ala Asn Leu Ala Trp Lys Leu Ala Gly Val Ile Gln Ala Thr Leu
305 310 315 320

Pro Met Asp Leu Leu Glu Ser Tyr Glu Ala Glu Arg Arg Pro Ile Ala
325 330 335

Ala Ala Ala Leu Arg Asn Thr Asp Leu Leu Phe Arg Phe Asn Thr Ala
340 345 350

Ser Gly Pro Ile Gly Arg Leu Ile His Trp Ile Gly Leu Gln Ala Thr
355 360 365

Arg Ala Pro Tyr Val Ala Gln Lys Val Val Ser Ala Leu Ala Gly Glu
370 375 380

Gly Val Arg Tyr Asp Ser Val Arg Arg Arg Gly Asp His Arg Leu Val
385 390 395 400

Gly Arg Arg Leu Pro Leu Leu Ser Leu Leu Pro Glu Gly Glu Arg Leu
405 410 415

Pro Arg Gln Ser Leu Thr Gln Leu Leu Arg Ala Gly Arg Phe Val Leu
420 425 430

Val His His Arg Ala Lys Ala Leu Ala Ala Asp Leu Arg Arg Asp Phe
435 440 445

Pro Gly Leu Gln Thr Ala Ser Ile Cys Glu Asp Ser His Asn Asn Ser
450 455 460

Leu Ser Ala Gly Glu Gly Val Ile Val Arg Pro Asp Gly Val Val Ile
465 470 475 480

Trp Val Gly Lys Lys Ser Thr Leu Ala Lys Glu Arg Leu Gly Glu Trp
485 490 495

Leu Leu Asp Asp Ser Lys Ser Ala Arg Gln Ser Leu Thr
500 505

<210> SEQ ID 12
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<212> TYPE: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 12

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20 25 30

Arg Ser Asp Ala Lys Asn Asn Val Val Phe Leu Leu Ala Asp Gly Glu
35 40 45

Glu Leu Gly Leu Leu Gly Ala Glu His Tyr Val Ser Gln Leu Ser Thr
50 55 60

Pro Glu Arg Glu Ala Ile Arg Leu Val Leu Asn Phe Glu Ala Arg Gly
65 70 75 80

Asn Gln Gly Ile Pro Leu Leu Phe Glu Thr Ser Gln Lys Asp Tyr Ala
85 90 95

Leu Ile Arg Thr Val Asn Ala Gly Val Arg Asp Ile Ile Ser Phe Ser
100 105 110

Phe Thr Pro Leu Ile Tyr Asn Met Leu Gln Asn Asp Thr Asp Phe Thr
115 120 125

Val Phe Arg Lys Lys Asn Ile Ala Gly Leu Asn Phe Ala Val Val Glu
130 135 140

Gly Phe Gln His Tyr His His Met Ser Asp Thr Val Glu Asn Leu Gly
145 150 155 160

Pro Glu Thr Leu Phe Arg Tyr Gln Lys Thr Val Arg Glu Val Gly Asn
165 170 175

His Phe Ile Gln Gly Ile Asp Leu Ser Ser Leu Ser Ala Asp Glu Asp
180 185 190

Ala Thr Tyr Phe Pro Leu Pro Gly Gly Thr Leu Leu Val Leu Asn Leu
195 200 205

Pro Thr Leu Tyr Ala Leu Gly Met Gly Ser Phe Val Leu Cys Gly Leu
210 215 220

Trp Ala Gln Arg Cys Arg Thr Arg Arg Gln His Gln Gly Lys Asn Cys
225 230 235 240

Val Leu Arg Pro Met Ala Ile Ala Leu Leu Gly Ile Ala Cys Ala Ala
245 250 255

Leu Val Phe Tyr Val Pro Ser Ile Ala Tyr Leu Phe Val Ile Pro Ser
260 265 270

Leu Leu Leu Ala Cys Ala Met Leu Ser Arg Ser Leu Phe Ile Ser Tyr
275 280 285

Ser Ile Met Leu Leu Gly Ala Tyr Ala Cys Gly Ile Leu Tyr Ala Pro
290 295 300

Ile Val Tyr Leu Ile Ser Ser Gly Leu Lys Met Pro Phe Ile Ala Gly
305 310 315 320

Val Ile Ala Leu Leu Pro Leu Cys Leu Leu Ala Val Gly Leu Ala Gly
325 330 335

Val Ile Ala Arg Ser Arg Asp Cys Arg Thr Cys Asp
340 345

<210> SEQ ID 13
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<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 13

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            20                  25                  30

Val Val Leu Phe Thr Asp Pro Ala Leu Val Cys Gln Gln Ile Glu Asp
        35                  40                  45

Ser Asp Ala Asp Leu Val Ile Cys Pro Phe Leu Lys Asp Arg Val Pro
    50                  55                  60

Gln Gln Leu Trp Ser Asn Leu Glu Arg Pro Val Val Ile Ile His Pro
65                  70                  75                  80

Gly Ile Val Gly Asp Arg Gly Ala Ser Ala Leu Asp Trp Ala Ile Ser
                85                  90                  95

Gln Gln Val Gly Arg Trp Gly Val Thr Ala Leu Gln Ala Val Glu Glu
            100                 105                 110

Met Asp Ala Gly Pro Ile Trp Ser Thr Cys Glu Phe Asp Met Pro Ala
        115                 120                 125

Asp Val Arg Lys Ser Glu Leu Tyr Asn Gly Ala Val Ser Asp Ala Ala
    130                 135                 140

Leu Tyr Cys Ile Arg Asp Val Val Glu Lys Phe Ala Arg Val Phe Val
145                 150                 155                 160

Pro Val Pro Leu Asp Tyr Thr Gln Ala His Val Ile Gly Arg Leu Gln
                165                 170                 175

Pro Asn Met Thr Gln Ala Asp Arg Thr Phe Ser Trp Tyr Asp Cys Ala
            180                 185                 190

Arg Phe Ile Lys Arg Cys Ile Asp Ala Ala Asp Gly Gln Pro Gly Val
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195 200 205

Leu Ala Ser Ile Gln Gly Gly Gln Tyr Tyr Leu Tyr Asp Ala His Leu
210 215 220

Asp Ala Arg His Gly Thr Pro Gly Glu Ile Leu Ala Val Gln Asp Asp
225 230 235 240

Ala Val Leu Val Ala Ala Gly Asp Gln Ser Leu Trp Ile Gly Ser Leu
245 250 255

Lys Arg Lys Ala Arg Pro Gly Glu Glu Thr Phe Lys Leu Pro Ala Arg
260 265 270

His Val Leu Ala Glu Ala Leu Ala Asp Ile Pro Val Leu Asp Ser Ser
275 280 285

Ile Ala Asn Gln Met Phe Asp Glu Gln Ala Tyr Gln Pro Ile Arg Tyr
290 295 300

Arg Glu Ala Gly His Val Gly Glu Leu Thr Phe Glu Phe Tyr Asn Gly
305 310 315 320

Ala Met Ser Thr Glu Gln Cys Gln Arg Leu Val Ala Ala Leu Arg Trp
325 330 335

Ala Lys Thr Arg Asp Thr Gln Val Leu Val Ile Lys Gly Gly Arg Gly
340 345 350

Ser Phe Ser Asn Gly Val His Leu Asn Val Ile Gln Ala Ala Pro Val
355 360 365

Pro Gly Leu Glu Ala Trp Ala Asn Ile Gln Ala Ile Tyr Asp Val Cys
370 375 380

His Glu Leu Leu Thr Ala Arg Gln Leu Val Ile Ser Gly Leu Thr Gly
385 390 395 400

Ser Ala Gly Ala Gly Gly Val Met Leu Ala Leu Ala Ala Asp Ile Val
405 410 415

Leu Ala Arg Glu Ser Val Val Leu Asn Pro His Tyr Lys Thr Met Gly
420 425 430

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Leu Tyr Gly Ser Glu Tyr Trp Thr Tyr Ser Leu Pro Arg Ala Val Gly
        435                 440                 445

Ser Glu Val Ala His Gln Leu Thr Asp Ala Cys Leu Pro Ile Ser Ala
    450                 455                 460

Leu Gln Ala Glu Gln Tyr Gly Leu Val Gln Gly Ile Gly Pro Arg Cys
465                 470                 475                 480

Pro His Ala Phe Ser Arg Trp Leu Met Gln Gln Ala Ser Ser Ala Leu
                485                 490                 495

Thr Asp Glu Lys Tyr Ala Val Ala Arg Ala Arg Lys Ala Ala Leu Asp
            500                 505                 510

Ile Asp Gln Ile Thr Arg Cys Arg Glu Ala Glu Leu Ala Gln Met Gln
        515                 520                 525

Leu Asp Met Val His Asn Arg His Gln Phe Ala Glu Lys Cys Arg Asn
    530                 535                 540

Phe Val Leu Lys Arg Lys Thr Cys Gln Thr Pro Gln Arg Leu Met Ala
545                 550                 555                 560

Pro Trp Ala Val Ala Arg Glu Ala Ala Leu Val Gly
                565                 570
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<211> Lenght: 230
<212> Type: PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 14

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1               5                   10                  15

Ala Cys Leu Ala Ser Ile Gln Arg Ala Ile Ala His Pro Ala Leu Ala
            20                  25                  30

His Gln Gln Val Gln Leu Leu Val Val Leu Asp Ala Cys Ser Asp Glu
        35                  40                  45
```

```
Thr Ala Thr Arg Val Ser Ala Met Gly Val Ala Thr Leu Glu Val Ser
 50                  55                  60

Val Arg Asn Val Gly Lys Ala Arg Ala Leu Gly Ala Glu Arg Leu Leu
65                  70                  75                  80

Glu Val Gly Ala Gln Trp Leu Ala Phe Thr Asp Ala Asp Thr Val Val
                85                  90                  95

Pro Ala Asp Trp Leu Val Arg Gln Ile Gly Phe Gly Ala Asp Ala Val
            100                 105                 110

Cys Gly Thr Val Glu Val Asp Ser Trp Ser Glu Tyr Gly Glu Ser Val
        115                 120                 125

Arg Ser Arg Tyr Leu Glu Leu Tyr Gln Phe Thr Glu Asn His Arg His
    130                 135                 140

Ile His Gly Ala Asn Leu Gly Leu Ser Ala Asp Ala Tyr Arg Asn Ala
145                 150                 155                 160

Gly Gly Phe Gln His Leu Val Ala His Glu Asp Val Gln Leu Val Ala
                165                 170                 175

Asp Leu Glu Arg Ile Gly Ala Arg Ile Val Trp Thr Ala Thr Asn Pro
            180                 185                 190

Val Val Thr Ser Ala Arg Arg Asp Tyr Lys Cys Arg Gly Gly Phe Gly
        195                 200                 205

Glu Tyr Leu Ala Ser Leu Val Ala Glu Gly Thr Arg Glu His Ser Pro
    210                 215                 220

Ala His Ala Pro Ile Gly
225                 230
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<210> SEQ ID 15
<211> Lenght: 348
<212> Type. PRT
<213> Organism: Pseudomonas fluorescens A2-2
<400> SEQUENCE: 15

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1               5                  10                  15

Phe Thr Thr Pro Gly Gly Val Phe His Asp Ala Val Lys Asp Val Met
            20                  25                  30

Gln Thr Ser Asn Met Leu Ala Asn Thr Ala Thr Thr Ile Glu Gln Ala
        35                  40                  45

Arg Lys Leu Gly Val Lys Ile Ile His Leu Pro Ile Arg Phe Ala Asp
    50                  55                  60

Gly Tyr Pro Glu Leu Thr Leu Arg Ser Tyr Gly Ile Leu Lys Gly Val
65                  70                  75                  80

Ala Asp Gly Ser Ala Phe Arg Ala Gly Ser Trp Gly Ala Glu Ile Thr
                85                  90                  95

Asp Ala Leu Lys Arg Asp Pro Thr Asp Ile Val Ile Glu Gly Lys Arg
            100                 105                 110

Gly Leu Asp Ala Phe Ala Thr Thr Gly Leu Asp Leu Val Leu Arg Asn
        115                 120                 125

Asn Gly Ile Gln Asn Leu Val Val Ala Gly Phe Leu Thr Asn Cys Cys
    130                 135                 140

Val Glu Gly Thr Val Arg Ser Gly Tyr Glu Lys Gly Tyr Asp Val Val
145                 150                 155                 160

Thr Leu Thr Asp Cys Thr Ala Thr Phe Ser Asp Glu Gln Gln Arg Ala
                165                 170                 175

Ala Glu Gln Phe Thr Leu Pro Met Phe Phe Ala Asn Pro Ala Thr His
            180                 185                 190

Arg Val Ser Ala Ser Thr Glu Arg Arg Ile Lys Lys Ala Ala Thr Pro
        195                 200                 205

Ala Glu Ser Pro Leu Phe Cys Leu Gly His Ser Val Gly Ala Tyr Cys
    210                 215                 220
```

Ile Ser Pro Phe Pro Asn Asp Gln Ser Ser Arg Phe Thr Ser Thr Arg
225 230 235 240

Leu Ile His Thr Ser Ser Leu Arg Ser Pro Val Leu Ala Trp Met Pro
245 250 255

Ser Ala Met Asn Leu Lys Ala Phe Phe Thr Ser Met Leu Arg Pro Ala
260 265 270

Phe His Val Thr Trp Ile Asn Thr Ile Leu Gly Val Val Thr Pro Arg
275 280 285

Tyr Pro Ala Ala Gly Thr Ser Ser Ser Leu Ala Trp Arg Leu Met Ile
290 295 300

Trp Asn Leu Ser Cys Ser Gly Thr Leu Ala Thr Leu Val Ile Ala Ala
305 310 315 320

Tyr Thr Thr Ser Pro Met Ala Val Ala Val Ser Val Glu Val Ser Ala
325 330 335

Ala Arg Ser Ile Arg Thr Lys Gly Met Asp Lys Ser
340 345

CLAIMS:

1. A gene cluster having open reading frames which encode polypeptides sufficient to direct the synthesis of a safracin molecule.

2. A nucleic acid sequence comprising:

a) a nucleic acid sequence encoding at least one non-ribosomal peptide synthetase which catalyse at least one step of the biosynthesis of safracins;

b) a nucleic acid sequence which is complementary to the sequence in a); or

c) variants or portions of the sequences of a) or b).

3. The nucleic acid sequence according to claim 2 which comprises SEQ ID NO:1, variants or portions thereof.

4. The nucleic acid sequence according to claim 2 which comprises at least one of the *sacA, sacB, sacC, sacD, sacE, sacF, sacG, sacH, sacI, sacJ, orf1, orf2, orf3* or *orf4* genes, including variants or portions thereof.

5. The nucleic acid sequence according to claim 2 wherein the nucleic acid encodes a polypeptide which is at least 30% identical in amino acid sequence to a polypeptide encoded by any of the safracin gene cluster open reading frames *sacA* to *sacJ* and *orf1* to *orf4* (SEQ ID NO:1 and genes encoded in SEQ ID NO:1) or encoded by a variant or portion thereof.

6. The nucleic acid sequence according to claim 2 which encodes for any of SacA, SacB, SacC, SacD, SacE, SacF, SacG, SacH, SacI, SacJ, Orf1, Orf2, Orf3 or Orf4 proteins (SEQ ID NO:2-15), and variants, mutants or portions thereof.

7. The nucleic acid sequence according to claim 2 which encodes a peptide synthetase, a L-Tyr derivative hidroxylase, a L-Tyr derivative methylase, a L-Tyr O-methylase, a methyl-transferase or a monooxygenase or a safracin resistance protein.

8. The nucleic acid sequence according to any one of claims 3-6 wherein the portion is at least 50 nucleotides in length.

9. The nucleic acid sequence according to claim 8 wherein the portion is in the range between 100 to 5000 nucleotides in length.

10. The nucleic acid sequence according to claim 8 wherein the portion is in the range between 100 to 2500 nucleotides in length.

11. A hybridization probe comprising a nucleic acid sequence according to any one of the preceding claims.

12. The hybridization probe according to claim 11 which comprises a sequence of at least 10 nucleotide residues.

13. The hybridization probe according to claim 11 which comprises a sequence between 25 to 60 nucleotide residues.

14. Use of a hybridization probe according to any one of claims 11-13 in the detection of a safracin or ecteinascidin gene.

15. The use according to claim 14 wherein the gene detection is conducted in *Ecteinascidia turbinata*.

16. A polypeptide encoded by a nucleic acid sequence of any one of claims 2-10.

17. The polypeptide according to claim 16 which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2-15.

18. A vector comprising a nucleic acid sequence of any one of claims 2-10.

19. The vector according to claim 18 which is an expression vector.

20. The vector according to claim 18 which is a cosmid.

21. A host cell transformed with one or more of the nucleic acid sequences of any one of claims 2-10.

22. A host cell comprising a vector of any one of claims 18-20.

23. The host cell according to claim 22 wherein the host cell is transformed with an exogenous nucleic acid comprising a gene cluster encoding polypeptides sufficient to direct the synthesis of a safracin.

24. The host cell according to claims 22 or 23 which is a microorganism.

25. The host cell according to claim 24 which is a bacterium.

26. A recombinant bacterial host cell in which at least a portion of a nucleic acid sequence of any one of claims 2-10 is disrupted to result in a recombinant host cell that produces altered levels of safracin compound or safracin analogue, relative to a corresponding nonrecombinant bacterial host cell.

27. The recombinant cell of claim 26, wherein the disrupted nucleic acid sequence is endogenous.

28. A method of producing a safracin compound or safracin analogue comprising fermenting an organism in which the copy number of the gene cluster of claim 1 has been increased.

29. A method of producing a safracin compound or safracin analogue comprising fermenting an organism in which expression of genes encoding polypeptides sufficient to direct the synthesis of a safracin or safracin analogue has been modulated by manipulation or replacement of one or more genes or sequence responsible for regulating such expression.

30. A method of producing a safracin compound or safracin analogue comprising contacting a compound that is a substrate for a polypeptide encoded by one or more of the open reading frames of the safracin biosynthesis gene cluster of claim 1 with said polypeptide, wherein the polypeptide chemically modifies the compound.

31. The method according to claims 28 or 29 wherein the organism is *Pseudomonas* sp.

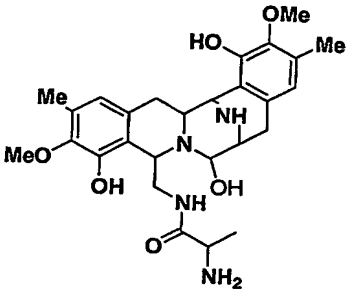
32. A composition comprising at least one nucleic acid sequence of any one of claims 2-10.

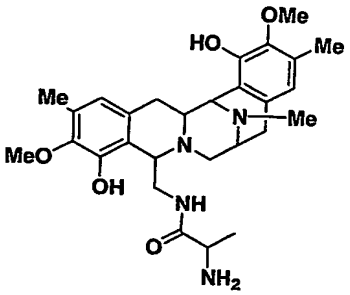
33 Use of a composition according to claim 32 for the combinatorial biosynthesis of one or more of non-ribosomal peptide synthetases, diketopiperazine rings and safracins.

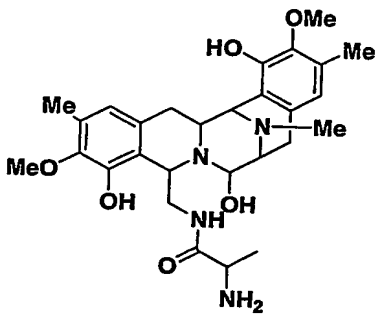
34. Use of P2, P14, analogs and derivatives thereof in combinatorial biosynthesis of one or more of non-ribosomal peptide synthetases, diketopiperazine rings and safracins.

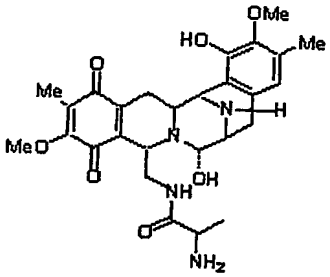
35. A safracin compound obtainable by a method according to any of claims 28-31.

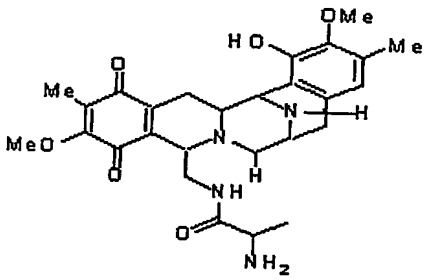
36. A safracin compound according to claim 35 wherein the compound has one of the following formulas

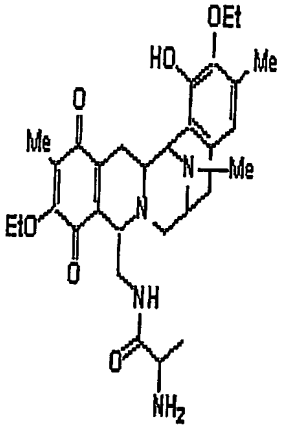


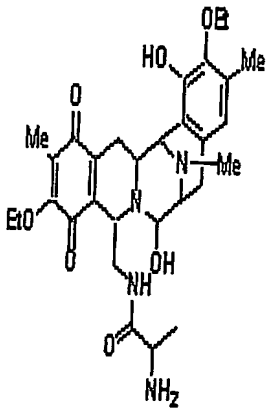












37. Use of a compound according to claims 35 or 36 as an antitumor agent.

38. Use of a compound according to claims 35 or 36 in the manufacture of a medicament for the treatment of cancer.

39. Use of a compound according to claims 35 or 36 as an antimicrobial agent.

40. Use of a compound according to claims 35 or 36 in the manufacture of a medicament for the treatment of microbial infections.

41. A pharmaceutical composition comprising a compound according to claims 35 or 36 and a pharmaceutically acceptable diluent, carrier or excipient.

42. Use of a compound according to claims 35 or 36 in the synthesis of ecteinascidin compounds.

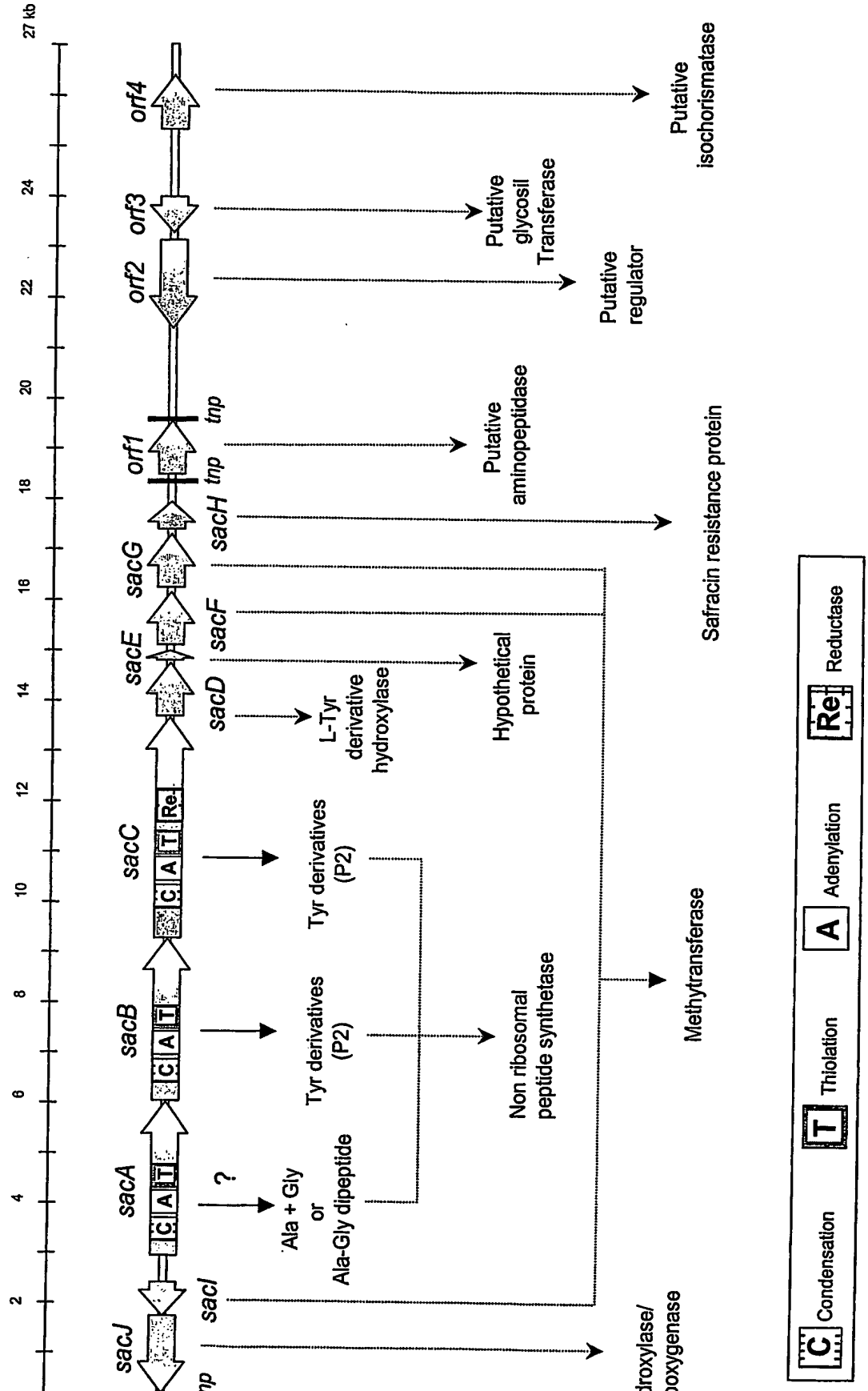
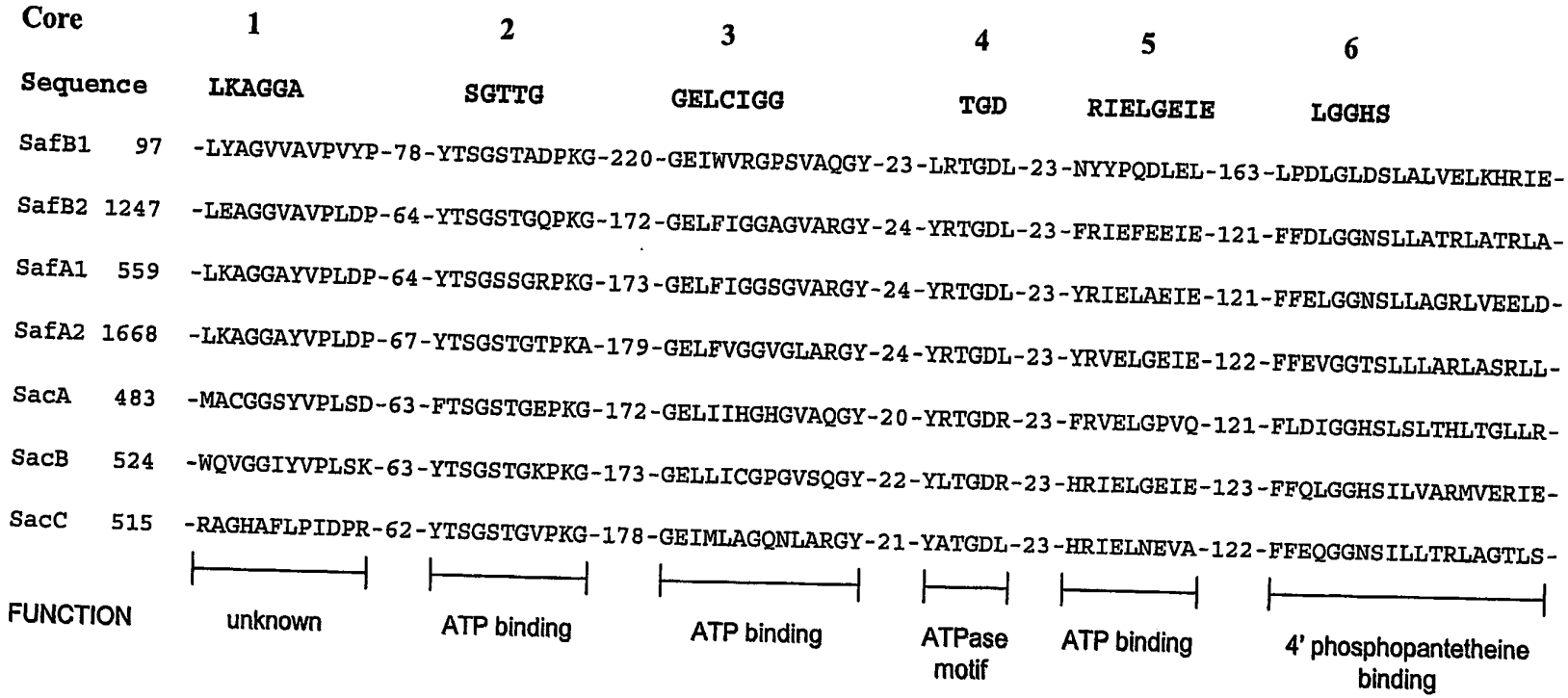

0
2
4
6
8
10
12
14
16
18
20
22
24
27 kb
tnp
sacJ
sacI
sacA
C A T
?
Ala + Gly
or
Ala-Gly dipeptide
sacB
C A T
Tyr derivatives
(P2)
sacC
C A T Re
Tyr derivatives
(P2)
sacD
L-Tyr
derivative
hydroxylase
sacE
Hypothetical
protein
sacF
sacG
Non ribosomal
peptide synthetase
sacH
Methytransferase
tnp
orf1
Safracin resistance protein
tnp
Putative
aminopeptidase
orf2
Putative
regulator
orf3
Putative
glycosil
Transferase
orf4
Putative
isochorismatase
Hydroxylase/
monooxygenase
C Condensation
T Thiolation
A Adenylation
Re Reductase

Figure 1


Core
1
2
3
4
5
6
Sequence
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SGTTG
GELCIGG
TGD
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LGGHS
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SafB2 1247 -LEAGGVAVPLDP-64-YTSGSTGQPKG-172-GELFIGGAGVARGY-24-YRTGDL-23-FRIEFEEIE-121-FFDLGGNSLLATRLATRLA-
SafA1 559 -LKAGGAYVPLDP-64-YTSGSSGRPKG-173-GELFIGGSGVARGY-24-YRTGDL-23-YRIELAEIE-121-FFELGGNSLLAGRLVEELD-
SafA2 1668 -LKAGGAYVPLDP-67-YTSGSTGTPKA-179-GELFVGGVGLARGY-24-YRTGDL-23-YRVELGEIE-122-FFEVGGTSLLLARLASRLL-
SacA 483 -MACGGSYVPLSD-63-FTSGSTGEPKG-172-GELIIHGHGVAQGY-20-YRTGDR-23-FRVELGPVQ-121-FLDIGGHSLSLTHLTGLLR-
SacB 524 -WQVGGIYVPLSK-63-YTSGSTGKPKG-173-GELLICGPGVSQGY-22-YLTGDR-23-HRIELGEIE-123-FFQLGGHSILVARMVERIE-
SacC 515 -RAGHAFLPIDPR-62-YTSGSTGVPKG-178-GEIMLAGQNLARGY-21-YATGDL-23-HRIELNEVA-122-FFEQGGNSILLTRLAGTLS-
FUNCTION
unknown
ATP binding
ATP binding
ATPase motif
ATP binding
4' phosphopantetheine binding

Figure 2

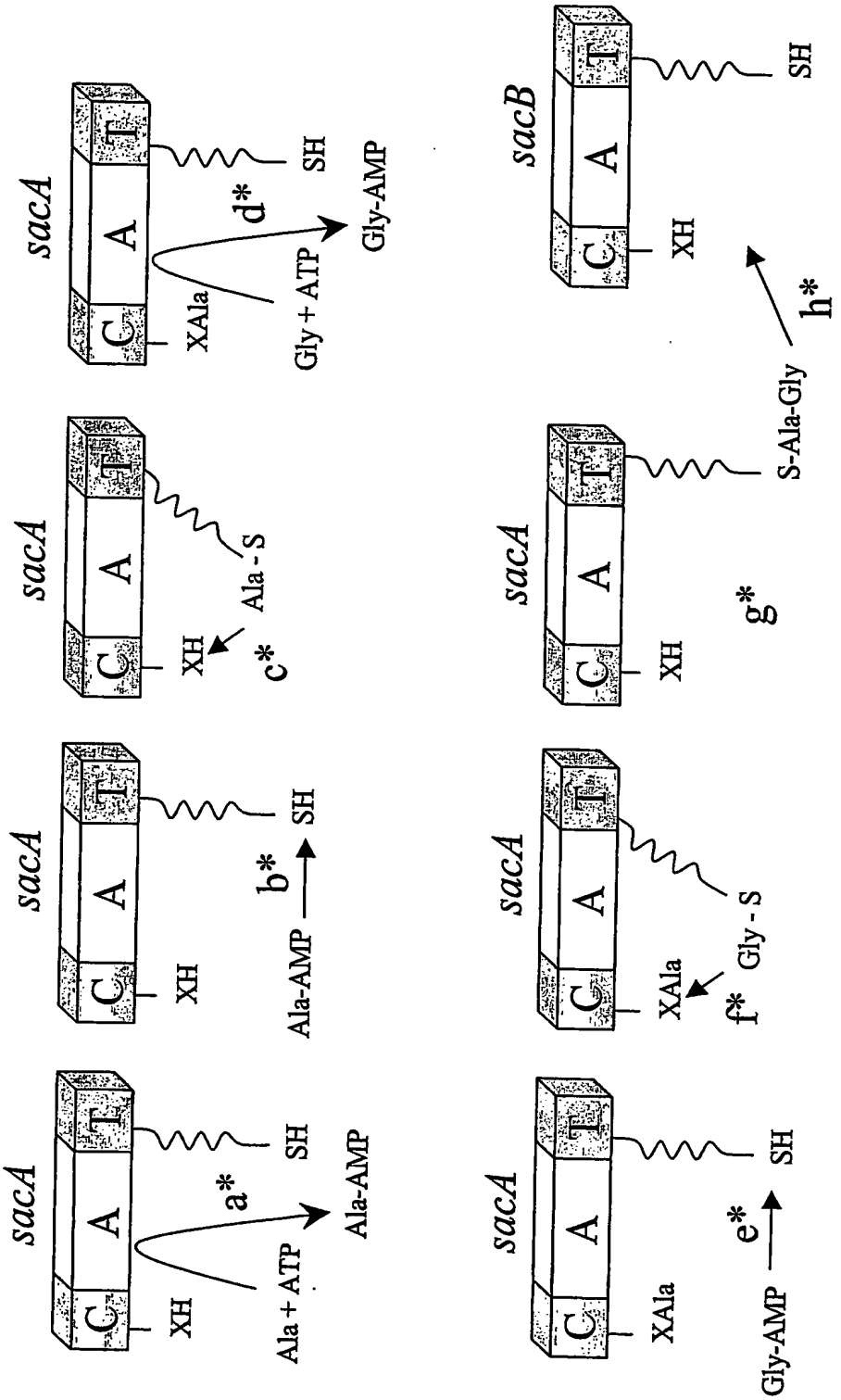
sacA
C
A
T
XH
Ala + ATP
a*
SH
Ala-AMP
sacA
C
A
T
XH
Ala-AMP
b*
SH
sacA
C
A
T
XH
c*
Ala - S
sacA
C
A
T
XAla
Gly + ATP
d*
SH
Gly-AMP
sacA
C
A
T
XAla
Gly-AMP
e*
SH
sacA
C
A
T
XAla
f*
Gly - S
sacA
C
A
T
XH
g*
S-Ala-Gly
sacB
C
A
T
XH
h*
SH

Figure 3

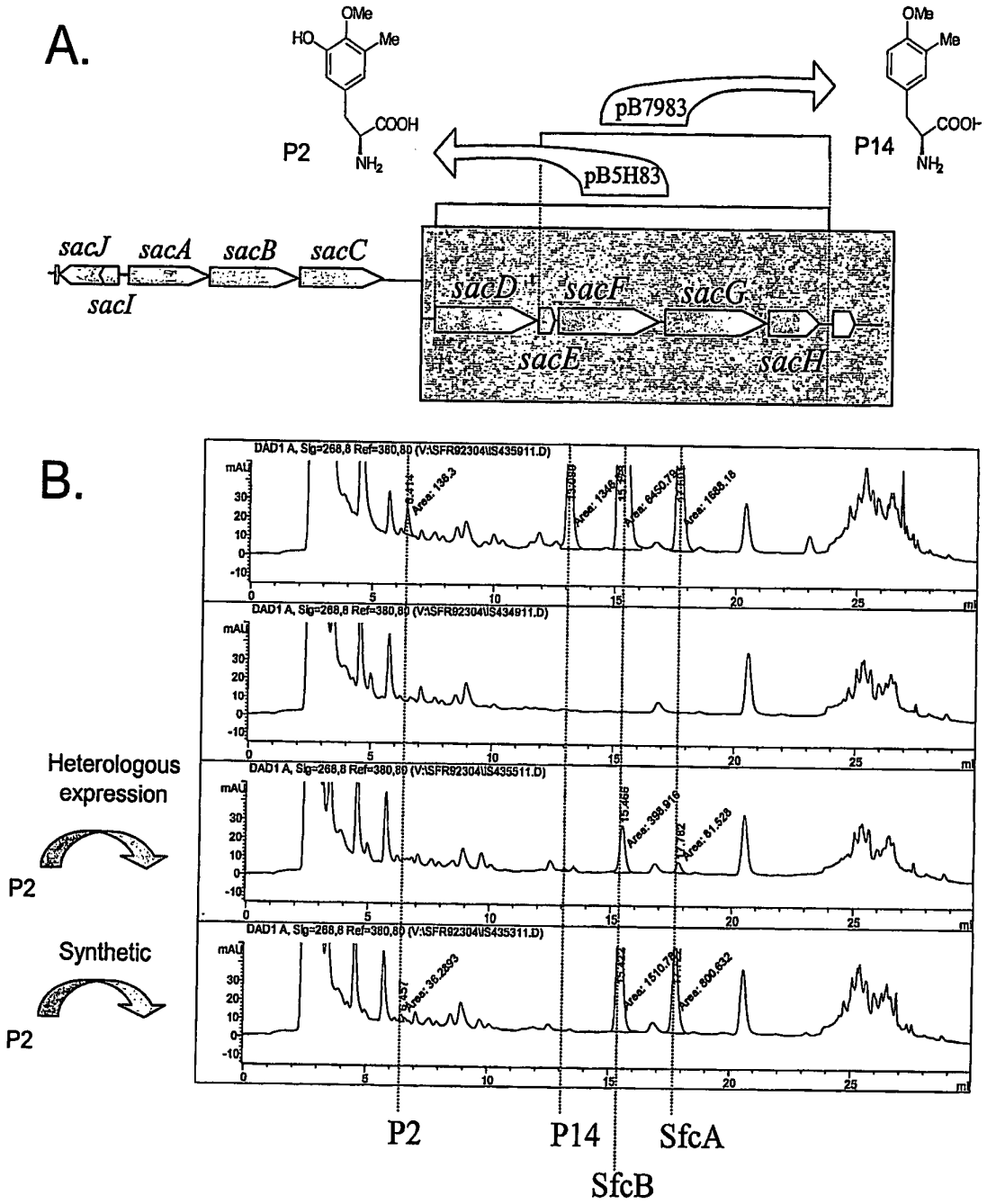
A.
OMe
HO
Me
COOH
NH2
P2
pB7983
pB5H83
OMe
Me
COOH
NH2
P14
sacJ
sacA
sacB
sacC
sacI
sacD
sacF
sacG
sacE
sacH
B.
Heterologous expression
P2
Synthetic
P2
P2
P14
SfcA
SfcB

Figure 4

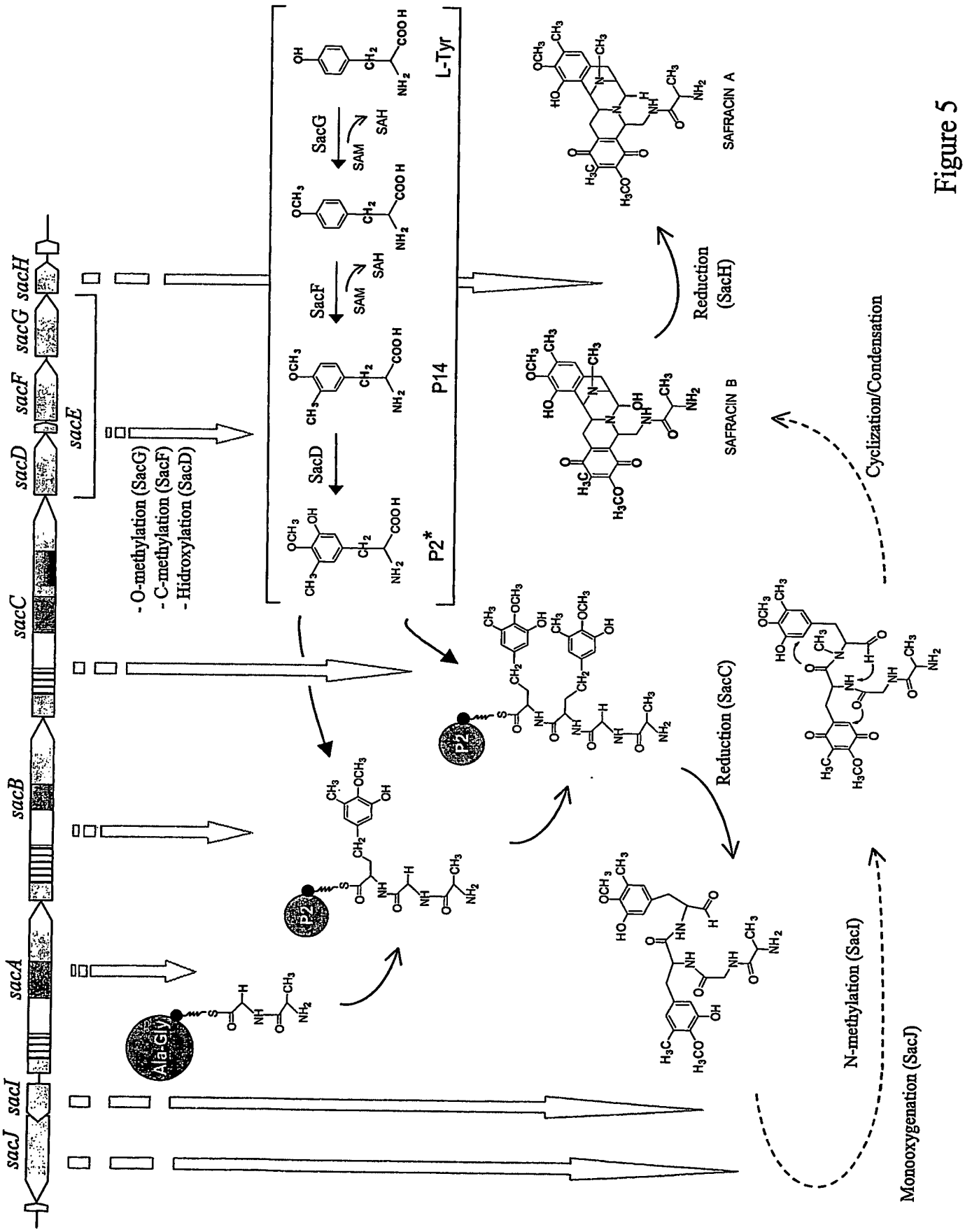
sacJ
sacI
sacA
sacB
sacC
sacD
sacE
sacF
sacG
sacH
- O-methylation (SacG)
- C-methylation (SacF)
- Hidroxylation (SacD)
L-Tyr
SacG
SacF
SacD
SAM
SAH
P14
P2*
P2
Ala-Gly
Reduction (SacC)
Reduction (SacH)
SAFRACIN A
SAFRACIN B
Cyclization/Condensation
N-methylation (SacI)
Monooxygenation (SacJ)

Figure 5

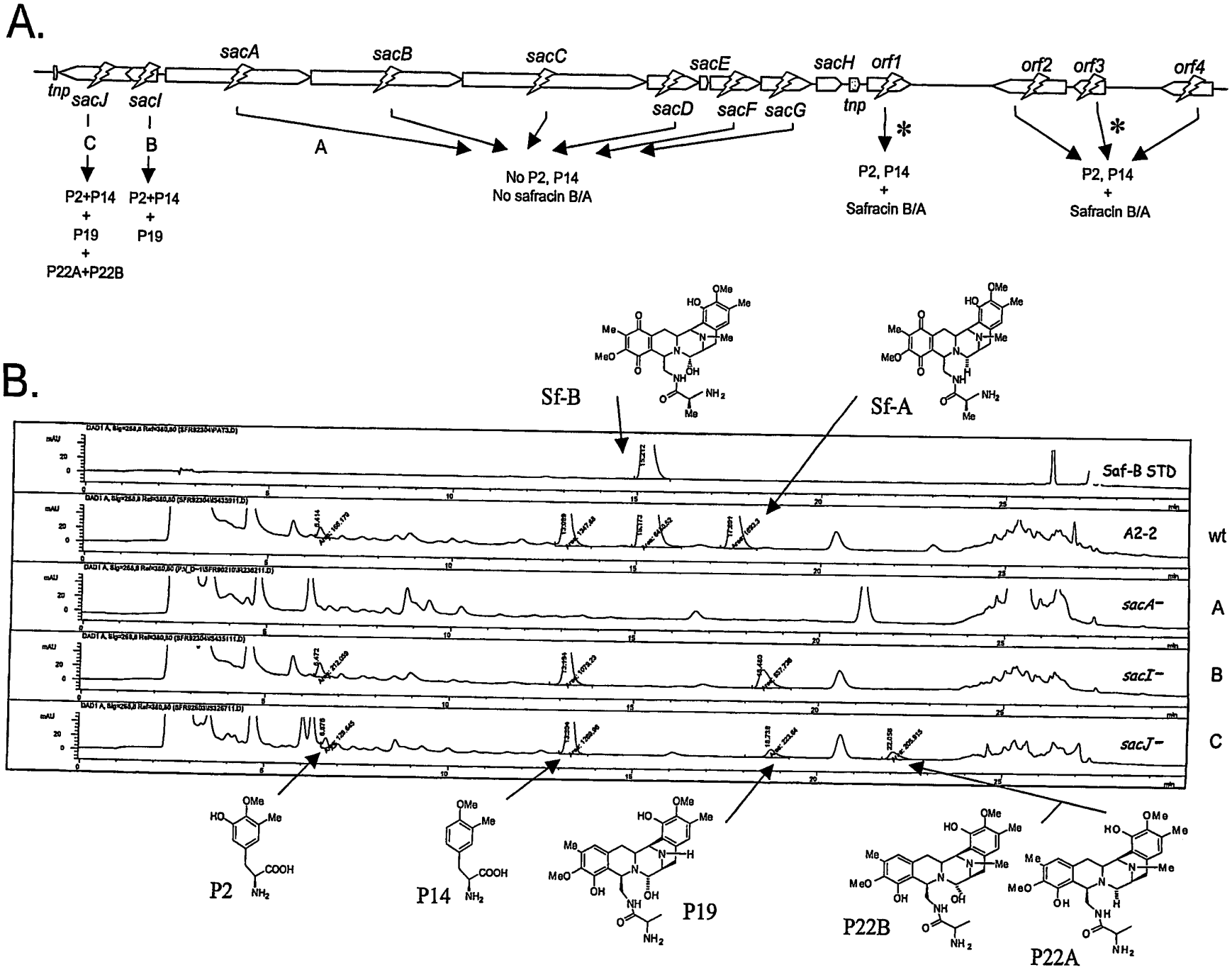
A.
sacA
sacB
sacC
sacE
sacH
orf1
orf2
orf3
orf4
tnp
sacJ
sacI
sacD
sacF
sacG
tnp
C
B
A
P2+P14
+
P19
+
P22A+P22B
P2+P14
+
P19
No P2, P14
No safracin B/A
P2, P14
+
Safracin B/A
P2, P14
+
Safracin B/A
B.
Sf-B
Sf-A
Saf-B STD
A2-2
wt
sacA-
A
sacI-
B
sacJ-
C
P2
P14
P19
P22B
P22A

Figure 6

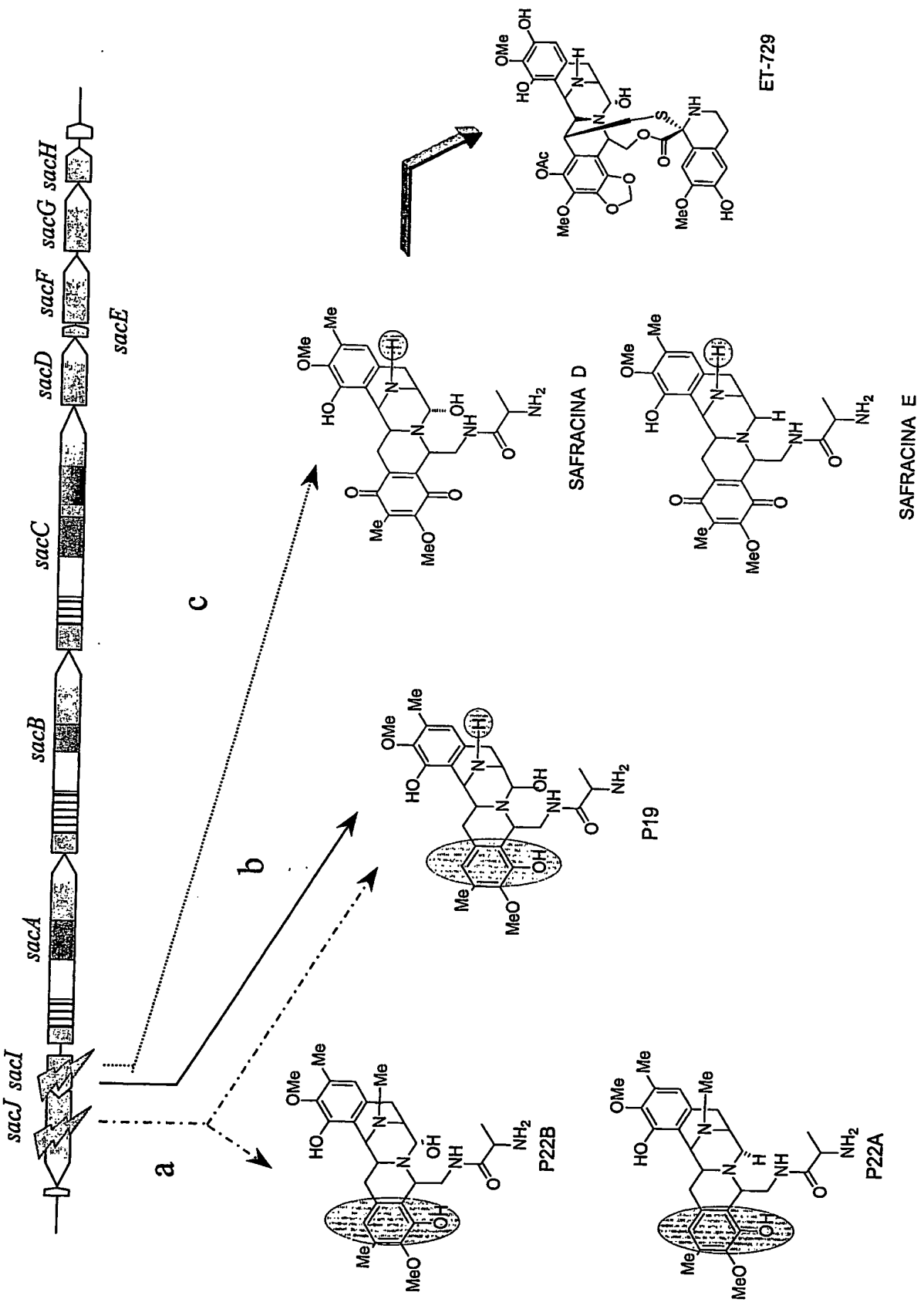
sacJ sacI
sacA
sacB
sacC
sacD
sacF
sacG sacH
sacE
a
b
c
P22B
P19
P22A
SAFRACINA D
SAFRACINA E
ET-729

Figure 7

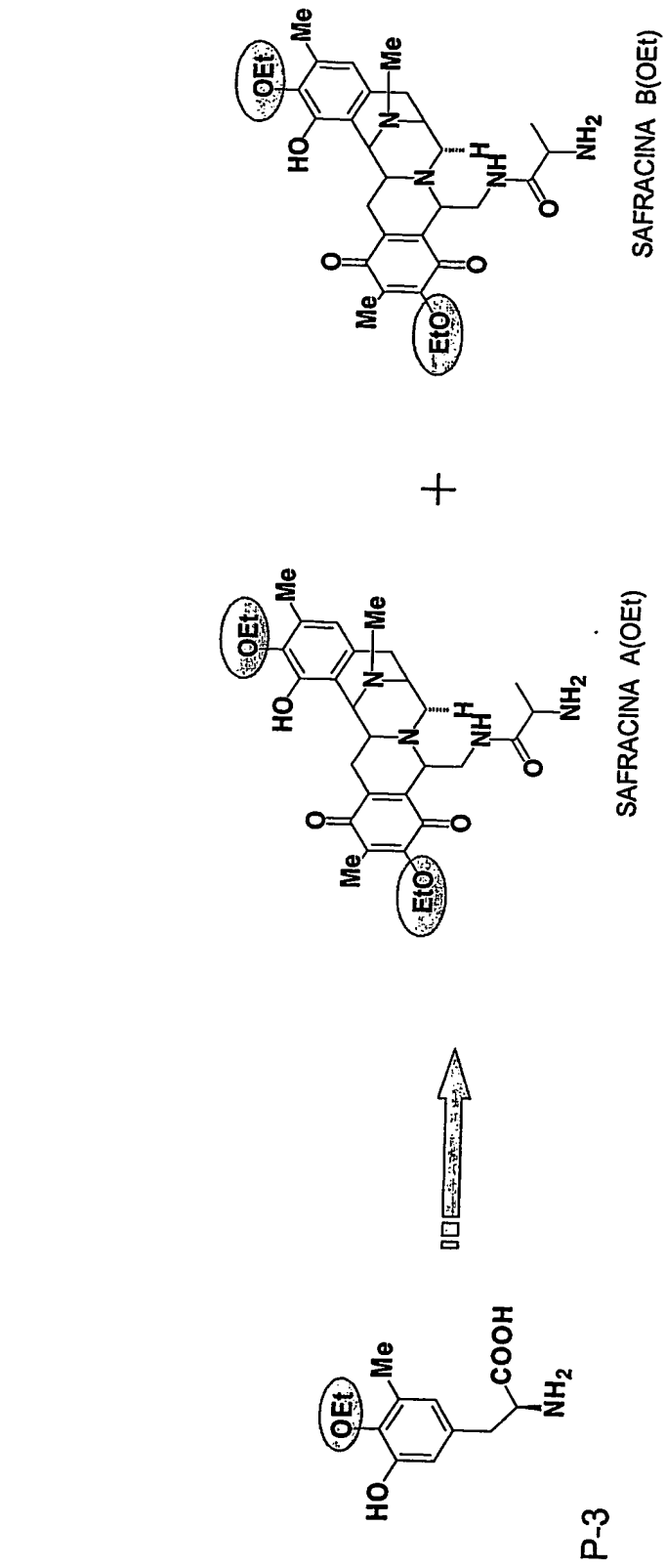

Figure 8

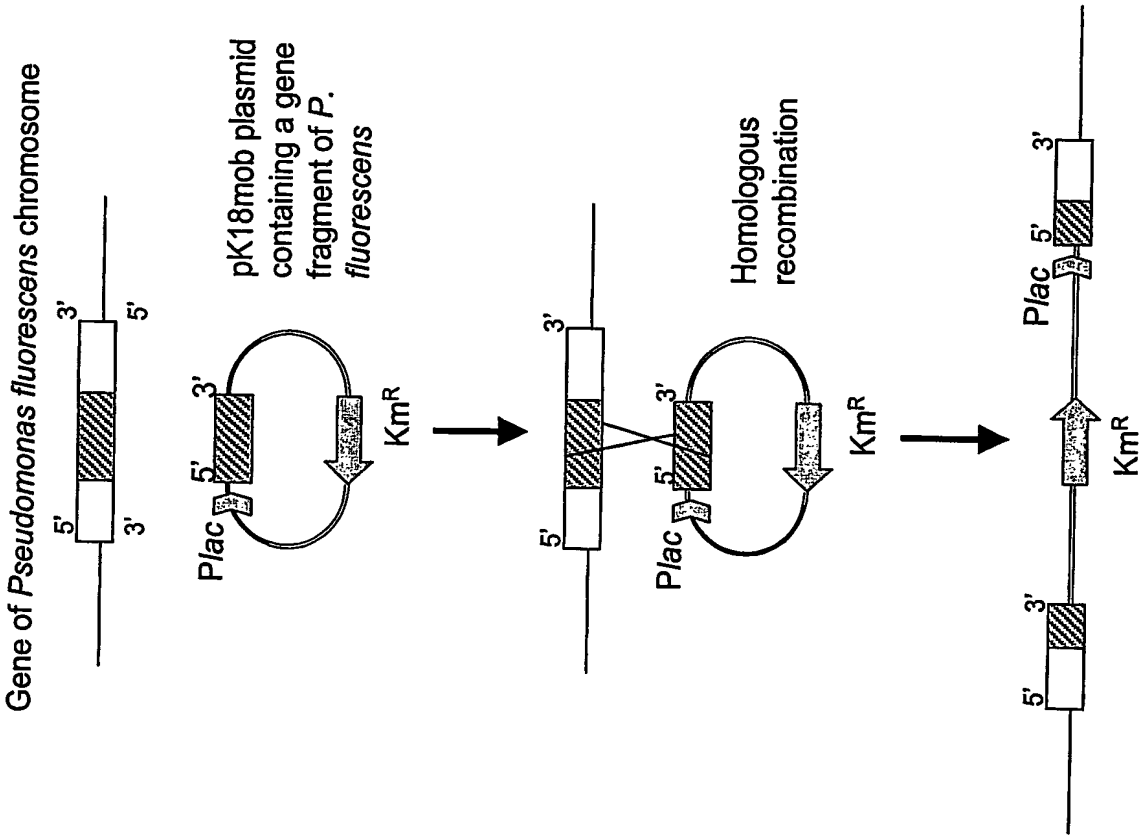
Gene of *Pseudomonas fluorescens* chromosome
3'
5'
5'
3'
pK18mob plasmid containing a gene fragment of *P. fluorescens*
3'
5'
Plac
Km^R
Homologous recombination
3'
5'
3'
5'
Plac
Km^R
3'
5'
Plac
5'
3'
Km^R
3'
5'

Figure 9

INTERNATIONAL SEARCH REPORT

PCT/GB 03/05563

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/52 C12N9/00 C12N9/02 C12N9/10 C07K14/21
C12Q1/68 C12N15/63 C12N15/53 C12N15/54 C12P17/12
C07D471/22 C07D471/18 A61K31/4995 A61P35/00 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q C12P C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, EMBL, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 055 299 A (YOSHITOMI PHARMACEUTICAL INDUSTRIES, LTD.) 7 July 1982 (1982-07-07) cited in the application	35,37-41
A	the whole document	36
A	MOHAMED A. MARAHIEL: "Protein templates for the biosynthesis of peptide antibiotics" CHEMISTRY AND BIOLOGY, vol. 4, August 1997 (1997-08), pages 561-567, XP000915211 cited in the application the whole document	1-33
X	WO 00/69862 A (PHARMA MAR, S.A.) 23 November 2000 (2000-11-23) cited in the application the whole document	35,37-42

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 June 2004	09/06/2004
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Montero Lopez, B

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

PCT/GB 03/05563

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANDREAS POSPIESCH ET AL.: "Two multifunctional peptide synthetases and a O-methyltransferase are involved in the biosynthesis of the DNA-binding antibiotic and antitumour agent saframycin Mx1 from Myxococcu xanthus" MICROBIOLOGY, vol. 142, 1996, pages 741-746, XP001118419 the whole document	1-27

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/GB 03/05563

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0055299 A	07-07-1982	JP	1300998 C	14-02-1986
		JP	57018633 A	30-01-1982
		JP	60020000 B	18-05-1985
		DE	3168135 D1	21-02-1985
		EP	0055299 A1	07-07-1982
		WO	8200146 A1	21-01-1982
		US	4440752 A	03-04-1984
		ZA	8104303 A	28-07-1982
WO 0069862 A	23-11-2000	AU	4597300 A	05-12-2000
		BG	106216 A	30-08-2002
		BR	0010559 A	02-07-2002
		CA	2372058 A1	23-11-2000
		CN	1360588 T	24-07-2002
		CZ	20014102 A3	12-06-2002
		EP	1185536 A2	13-03-2002
		WO	0069862 A2	23-11-2000
		HU	0201188 A2	29-07-2002
		JP	2002544280 T	24-12-2002
		NO	20015547 A	14-01-2002
		NO	20025186 A	14-01-2003
		NO	20025445 A	14-01-2003
		PL	353002 A1	22-09-2003
		SK	16502001 A3	04-06-2002
		TR	200103273 T2	22-04-2002
		US	2004002602 A1	01-01-2004

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* WPP287203

Box No. I TITLE OF INVENTION

The Gene Cluster Involved in Safracin Biosynthesis and its Uses for Genetic Engineering

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Pharma Mar, S.A.
Calle de la Calera 3
Poligono Industrial de Tres Cantos
Tres Cantos,
Madrid, E-28760, Spain

Telephone No.

Facsimile No.

Teleprinter No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Velasco Iglesias, Ana
Polígono Industrial La Mina
Avda. de los Reyes, 1
Colmenar Viejo,
Madrid, 28770, Spain

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Ruffles, Graham Keith
Marks & Clerk
66-68 Hills Road
Cambridgeshire CB2 1LA
United Kingdom

Telephone No. 01223 345520

Facsimile No. 01223 365560

Teleprinter No.

Agent's registration No. with the Office

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

de la Calle, Fernando
c/o Pharma Mar, S.A.
Calle de la Calera 3
Polígono Industrial de Tres Cantos
Tres Cantos, Madrid, 28760, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Aparicio Pérez, Tomás
Polígono Industrial La Mina
Avda. de los Reyes, 1
Colmenar Viejo,
Madrid, 28770, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Schleissner Sánchez, Carmen
Polígono Industrial La Mina
Avda. de los Reyes, 1
Colmenar Viejo,
Madrid, 28770, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Acebo Páis, Paloma
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Colmenar Viejo,
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This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Rodríguez Ramos, Pilar
Polígono Industrial La Mina
Avda. de los Reyes, 1
Colmenar Viejo,
Madrid, 28770, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Reyes Benítez, Fernando
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Avda. de los Reyes, 1
Colmenar Viejo
Madrid, 28770, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Henríquez Pelaez, Rubén
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Colmenar Viejo
Madrid, 28770, Spain

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Ruffles, Graham Keith
66-68 Hills Road
Cambridgeshire CB2 1LA
United Kingdom

This person is:
- ☒ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: ES

State *(that is, country)* of residence: ES

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☒ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **MZ** Mozambique, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZM** Zambia, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

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☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **BG** Bulgaria, **CH & LI** Switzerland and Liechtenstein, **CY** Cyprus, **CZ** Czech Republic, **DE** Germany, **DK** Denmark, **EE** Estonia, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **HU** Hungary, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **RO** Romania, **SE** Sweden, **SI** Slovenia, **SK** Slovakia, **TR** Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GQ** Equatorial Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ **AE** United Arab Emirates
☒ **AG** Antigua and Barbuda
☒ **AL** Albania
☒ **AM** Armenia
☒ **AT** Austria
☒ **AU** Australia
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina
☒ **BB** Barbados
☒ **BG** Bulgaria
☒ **BR** Brazil
☒ **BY** Belarus
☒ **BZ** Belize
☒ **CA** Canada
☒ **CH & LI** Switzerland and Liechtenstein
☒ **CN** China
☒ **CO** Colombia
☒ **CR** Costa Rica
☒ **CU** Cuba
☒ **CZ** Czech Republic
☒ **DE** Germany
☒ **DK** Denmark
☒ **DM** Dominica
☒ **DZ** Algeria
☒ **EC** Ecuador
☒ **EE** Estonia
☒ **ES** Spain
☒ **FI** Finland
☒ **GB** United Kingdom
☒ **GD** Grenada
☒ **GE** Georgia
☒ **GH** Ghana
☒ **GM** Gambia
☒ **HR** Croatia
☒ **HU** Hungary
☒ **ID** Indonesia
☒ **IL** Israel
☒ **IN** India
☒ **IS** Iceland
☒ **JP** Japan
☒ **KE** Kenya
☒ **KG** Kyrgyzstan
☒ **KP** Democratic People's Republic of Korea
☒ **KR** Republic of Korea
☒ **KZ** Kazakhstan
☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho
☒ **LT** Lithuania
☒ **LU** Luxembourg
☒ **LV** Latvia
☒ **MA** Morocco
☒ **MD** Republic of Moldova
☒ **MG** Madagascar
☒ **MK** The former Yugoslav Republic of Macedonia
☒ **MN** Mongolia
☒ **MW** Malawi
☒ **MX** Mexico
☒ **MZ** Mozambique
☒ **NI** Nicaragua
☒ **NO** Norway
☒ **NZ** New Zealand
☒ **OM** Oman
☒ **PG** Papua New Guinea
☒ **PH** Philippines
☒ **PL** Poland
☒ **PT** Portugal
☒ **RO** Romania
☒ **RU** Russian Federation
☒ **SC** Seychelles
☒ **SD** Sudan
☒ **SE** Sweden
☒ **SG** Singapore
☒ **SK** Slovakia
☒ **SL** Sierra Leone
☒ **SY** Syrian Arab Republic
☒ **TJ** Tajikistan
☒ **TM** Turkmenistan
☒ **TN** Tunisia
☒ **TR** Turkey
☒ **TT** Trinidad and Tobago
☒ **TZ** United Republic of Tanzania
☒ **UA** Ukraine
☒ **UG** Uganda
☒ **US** United States of America
☒ **UZ** Uzbekistan
☒ **VC** Saint Vincent and the Grenadines
☒ **VN** Viet Nam
☒ **YU** Serbia and Montenegro
☒ **ZA** South Africa
☒ **ZM** Zambia
☒ **ZW** Zimbabwe

Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

☒ **BW** Botswana ☒ **EG** Egypt ☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

   (i) ***if more than two persons are to be indicated as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

   (ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

   (iii) *if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

   (iv) *if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

   (v) *if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication **"patent of addition,"** or **"certificate of addition,"** or if, in Box No. V, the name of the United States of America is accompanied by an indication **"continuation"** or **"continuation-in-part"**: in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

   (vi) *if, in Box No. VI, there are **more than five earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

2. *If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

Continuation of Box II
Ruffles, Graham Keith is co-applicant for SD (Sudan) only

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application:* regional Office	international application: receiving Office
item (1) 20 December 2002 (20/12/02)	0229793.5	GB		
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* ....

.....

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / .....

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application **contains:**

(a) the following number of **sheets in paper form:**

request (including declaration sheets)	:	7
description (excluding sequence listing part)	:	107
claims	:	7
abstract	:	1
drawings	:	9
Sub-total number of sheets	:	131
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:	
Total number of sheets	:	131

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*: .....

This international application is **accompanied by** the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

Number of items

1. ☒ fee calculation sheet :
2. ☐ original separate power of attorney :
3. ☐ original general power of attorney :
4. ☐ copy of general power of attorney; reference number, if any: ..... :
5. ☐ statement explaining lack of signature :
6. ☐ priority document(s) identified in Box No. VI as item(s): ..... :
7. ☐ translation of international application into *(language)*: ..... :
8. ☐ separate indications concerning deposited microorganism or other biological material :
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))
   - (i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application) :
   - (ii) ☐ *(only where check-box (b)(i) or (b)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* :
   - (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column :
10. ☒ other *(specify)*: Form 23/77. ..... :

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Ruffles Graham Keith

For receiving Office use only

1. Date of actual receipt of the purported international application:

2. Drawings:
   ☐ received:
   ☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International Application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: WPP287203

Applicant: Pharma Mar, S.A. et al

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . 55.00 [T]

2. SEARCH FEE . . . . . 640.00 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent to carry out the international search, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

Where item (b) of Box No. IX applies, enter **Sub-total number of sheets** } 131
Where item (b) of Box No. IX does not apply, enter **Total number of sheets** }

[b1] first 30 sheets . . . . . 278 [b1]

[b2] 101 (number of sheets in excess of 30) x 6 (fee per sheet) = 606 [b2]

[b3] additional component (only if sequence listing part of description is filed in computer readable form under Section 801(a)(i), or both in that form and on paper, under Section 801(a)(ii)):

400 x _____ (fee per sheet) = _____ [b3]

Add amounts entered at b1, b2 and b3 and enter total at B . . . . 884 [B]

Designation Fees

The international application contains All designations.

5 (number of designation fees payable *(maximum 5)*) x 60 (amount of designation fee) = 300 [D]

Add amounts entered at B and D and enter total at I . . . . . 1184 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . 22 [P]

5. TOTAL FEES PAYABLE . . . . . 1901 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below) ☐ postal money order ☐ cash ☐ coupons

☐ cheque ☐ bank draft ☐ revenue stamps ☐ other *(specify):*

AUTHORIZATION TO CHARGE (OR CREDIT) DEPOSIT ACCOUNT

(This mode of payment may not be available at all receiving Offices)

Receiving Office: RO/ GB

Deposit Account No.: D10176

Date: 18 December 2003

Name: L. Gannon

Signature: _____

☒ Authorization to charge the total fees indicated above.

☐ *(This check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* Authorization to charge any deficiency or credit any overpayment in the total fees indicated above.

☐ Authorization to charge the fee for priority document.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

RUFFLES, Graham, keith
Marks & Clerk
66-68 Hills Road
Cambridgeshire CB2 1LA
ROYAUME-UNI

RECEIVED
[illegible] 2004
L03836

Date of mailing *(day/month/year)* 08 July 2004 (08.07.2004)	
Applicant's or agent's file reference WPP287203	**IMPORTANT NOTICE**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB2003/005563	19 December 2003 (19.12.2003)	20 December 2002 (20.12.2002)

Applicant
PHARMA MAR, S.A. et al

1. Notice is hereby given that the International Bureau has **communicated**, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice:

   AU, AZ, BY, CH, CN, CO, DZ, EP, HU, JP, KG, KP, KR, MD, MK, MZ, RU, TM, US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

   AE, AG, AL, AM, AP, AT, BA, BB, BG, BR, BW, BZ, CA, CR, CU, CZ, DE, DK, DM, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, ID, IL, IN, IS, KE, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PG, PH, PL, PT, RO, SC, SD, SE, SG, SK, SL, SY, TJ, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-*bis*)).

3. Enclosed with this notice is a copy of the international application as published by the International Bureau on 08 July 2004 (08.07.2004) under No. WO 2004/056998

4. **TIME LIMITS for filing a demand for international preliminary examination and for entry into the national phase**

   The applicable time limit for entering the national phase will, **subject to what is said in the following paragraph,** be **30 MONTHS** from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of **19 months** from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

   In practice, **time limits other than the 30-month time limit** will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For **regular updates on the applicable time limits** (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at http://www.wipo.int/pct/en/index.html.

   For filing a **demand for international preliminary examination**, see the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

   It is the applicant's **sole responsibility** to monitor all these time limits.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Nora Lindner
Facsimile No.+41 22 740 14 35	Facsimile No.+41 22 338 89 65

Form PCT/IB/308 (April 2002)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 July 2004 (08.07.2004)

PCT

(10) International Publication Number
WO 2004/056998 A1

(51) International Patent Classification[7]: C12N 15/52, 9/00, 9/02, 9/10, C07K 14/21, C12Q 1/68, C12N 15/63, 15/53, 15/54, C12P 17/12, C07D 471/22, 471/18, A61K 31/4995, A61P 35/00, 31/04

(21) International Application Number:
PCT/GB2003/005563

(22) International Filing Date:
19 December 2003 (19.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0229793.5 20 December 2002 (20.12.2002) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: THE GENE CLUSTER INVOLVED IN SAFRACIN BIOSYNTHESIS AND ITS USES FOR GENETIC ENGINEERING

(57) Abstract: A gene cluster has open reading frames which encode polypeptides sufficient to direct the synthesis of a safracin molecule.

WO 2004/056998 A1